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Canadian Journal of Biochemistry and Physiology

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VOLUME 35 SEPTEMBER 1957 NUMBER 9

A COMPARISON OF THE CONCENTRATION OF C^{14} IN THE TISSUES OF PREGNANT AND NONPREGNANT FEMALE RATS FOLLOWING THE INTRAVENOUS ADMINISTRATION OF VITAMIN K_1-C^{14} AND VITAMIN K_3-C^{14} [1]

J. D. TAYLOR,[2] G. J. MILLAR, AND R. J. WOOD

Abstract

The C^{14} content was determined of the livers, spleens, skeletal muscle, blood, feces, and urine of both pregnant and nonpregnant female rats and of the placentas, fetal livers, fetuses, and amnionic fluids of pregnant rats following the intravenous administration of 5 mg./kg. of either vitamin K_1-C^{14} or vitamin K_3-C^{14}. The C^{14} concentrations of the livers of the rats given vitamin K_1 were about 24 times larger than those of animals that had received vitamin K_3-C^{14}. A fivefold difference in the same direction exists between the concentrations in the spleens of the two groups. The C^{14} levels for skeletal muscle, blood, placenta, fetal liver, and fetal tissue were of similar magnitude regardless of whether vitamin K_1 or vitamin K_3 was administered. Isotope dilution tests revealed that following intravenous administration of vitamin K_1-C^{14} the amount of radioactivity present as unchanged vitamin K_1-C^{14} was 12% for fetal tissue, 59% for placenta, and 120% for the maternal liver. The dry weights of the livers of pregnant rats were larger than those of nonpregnant rats and the increase was proportional to the live weight of the pregnant rat. No significant difference could be demonstrated in the percentage of the injected dose of vitamin K_1 deposited in the livers of pregnant or nonpregnant rats. The same was true for vitamin K_3-C^{14}. The results of this experiment indicate that vitamin K_3-C^{14} is not concentrated in the liver of the rat whereas vitamin K_1-C^{14} is. Furthermore, it would appear that both vitamin K_1 and vitamin K_3 can pass the placental barrier of the rat.

Introduction

An interesting approach to the study of the metabolism of vitamin K is afforded by the phenomena associated with the prolonged prothrombin time demonstrable in the plasma of the newborn. The "prothrombin activity" of the blood of the newborn infant is usually less than that of adults and appears to be uninfluenced by the antepartum administration of vitamin K to the mother (10, 6). An even greater prolongation of the prothrombin time of the

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Contribution from the Department of Physiology and Pharmacology and the Department of Chemistry, University of Saskatchewan, Saskatoon, Saskatchewan. This work was supported by the National Research Council, Ottawa, and the Atomic Energy Control Board.
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infant appears between the second and fourth day of life (8, 11). Owen and Hurn have shown that the postpartum prolongation of prothrombin time is due to a decrease in both prothrombin and proconvertin (factor VII) but not in Ac-globulin (9). This secondary prolongation in prothrombin time is influenced by the antepartum administration of vitamin K analogues to the mother (10) or postpartum administration of vitamin K_1 or synkavite to the infant (4).

The question naturally arises as to whether or not the fetus is supplied with vitamin K from the mother. Dam, Prange, and Søndergaard (3) reported that they were unable to detect vitamin K_1 in the placenta or fetus following intravenous administration of 100 mg./kg. of vitamin K_1 to the mother rat. The experiments reported here demonstrate a transfer of C^{14} to the placenta and fetus following the administration of either radioactive vitamin K_1-C^{14} or vitamin K_3-C^{14}. With the aid of isotope dilution techniques it was possible to show that unchanged vitamin K_1 was present in both the placenta and the fetus following vitamin K_1-C^{14} administration.

Experimental

Colloidal suspensions of 0.1% vitamin K_1-C^{14} or vitamin K_3-C^{14} were prepared in 5% Tween 80 for administration to animals. An equal weight of corn oil was added to solubilize the vitamin K_3-C^{14} before preparation of the suspension.

Female rats weighing 150–200 grams were examined daily by vaginal smear to demonstrate the estrus cycle, and a check for sperm in the vagina was made after the animals were mated. Eighteen days after the day of conception six pregnant rats were given an intravenous injection of 5 mg./kg. of vitamin K_1-C^{14} (specific activity 8.4×10^5 counts/min./mg.) and six pregnant rats were given an intravenous injection of 5 mg./kg. of vitamin K_3-C^{14} (specific activity 3.3×10^5 counts/min./mg.). Twenty four hours after the administration the animals were decapitated and their uteri were ligatured and removed. The tissues and fluids shown in Fig. 1 were assayed for C^{14}. The vitamin K-C^{14} equivalents were calculated from the specific activity of the dry tissue and the specific activity of the injected vitamin as follows:

$$\text{vitamin K-C}^{14}\text{ equivalent } (\mu\text{g./g.}) = \frac{\text{specific activity of dry tissue (counts/min./mg.)}}{\text{specific activity of vitamin K-C}^{14}\text{ (counts/min./mg.)}} \times \frac{10^6\ \mu\text{g.}}{1\ \text{g.}}.$$

This manner of expressing the concentration of radioactivity in tissues had the advantage of permitting a direct comparison although the specific activities and molecular weights of vitamin K-C^{14} preparations differed from one another. Twelve nonpregnant female rats were retained as controls. Six of these animals received 5 mg./kg. of the vitamin K_1-C^{14} and six received 5 mg./kg. of the vitamin K_3-C^{14}. In order to determine the amount of

radioactivity present as unchanged vitamin K_1-C^{14}, a pregnant rat was given 5 mg./kg. of vitamin K_1-C^{14} on the 18th day of gestation; it was sacrificed 24 hours later; and isotope dilution tests were performed on the the maternal liver and on the placental and fetal tissue by the method previously employed in this laboratory (13).

Results

The data presented in Fig. 1 and Table I show that, following the intravenous administration of either vitamin K_1-C^{14} or vitamin K_3-C^{14}, the placenta, fetus, fetal liver, and amnionic fluid contained C^{14} and that the vitamin K_1-C^{14} equivalents are of the same order of magnitude as the vitamin K_3-C^{14} equivalents. A comparison of data for animals given vitamin K_1-C^{14} with those given vitamin K_3-C^{14} showed that a marked concentration of C^{14} occurred in the livers and spleens of the animals given vitamin K_1-C^{14} whereas the concentration was much less marked in the animals given vitamin K_3-C^{14}. It is interesting to note that the concentration of C^{14} in the blood of animals that had received vitamin K_3-C^{14} was greater than the concentration in the blood of the rats that were given vitamin K_1-C^{14}. The same trend was apparent in the skeletal muscle of the two groups but the difference was not statistically significant. The vitamin K_1-C^{14} equivalents for the fetal livers were much lower than those for the maternal livers whereas when vitamin K_3-C^{14} was given, the maternal and fetal livers had similar vitamin K equivalents.

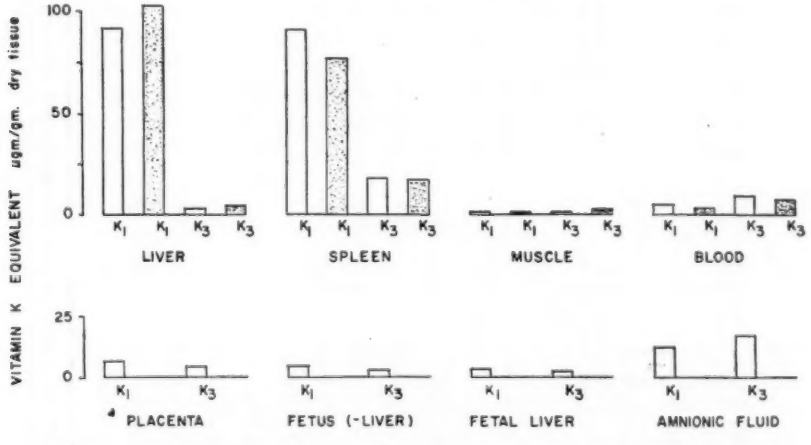

FIG. 1. A comparison of the mean vitamin K equivalents (μg./g. dry tissue) in the tissues of pregnant and nonpregnant female rats following the intravenous administration of vitamin K_1-C^{14} and vitamin K_3-C^{14}.
K_1 = 5 mg./kg. vitamin K_1-C^{14} injected intravenously.
K_3 = 5 mg./kg. vitamin K_3-C^{14} injected intravenously.
The open bars represent the pregnant rats whereas the stippled bars are for nonpregnant rats. Each bar represents the mean of six animals.

TABLE I

THE PERCENTAGE OF THE INJECTED DOSE OF VITAMIN K_1-C^{14} OR VITAMIN K_3-C^{14} AND THE VITAMIN K_1-C^{14} OR VITAMIN K_3-C^{14} EQUIVALENTS IN SOME RAT TISSUES FOLLOWING THE INTRAVENOUS ADMINISTRATION OF VITAMIN K_1-C^{14} OR VITAMIN K_3-C^{14} TO PREGNANT AND NONPREGNANT FEMALE RATS

	Vitamin K_1-C^{14} administered				Vitamin K_3-C^{14} administered			
	Six pregnant rats (mean ± standard error)		Six nonpregnant rats (mean ± standard error)		Six pregnant rats (mean ± standard error)		Six nonpregnant rats (mean ± standard error)	
Tissue	% of the injected dose	Vitamin K_1-C^{14} equivalent μg./g. dry tissue	% of the injected dose	Vitamin K_1-C^{14} equivalent μg./g. dry tissue	% of the injected dose	Vitamin K_3-C^{14} equivalent μg./g. dry tissue	% of the injected dose	Vitamin K_3-C^{14} equivalent μg./g. dry tissue
Liver	18.4 ±1.11	91.8 ± 5.64	19.3 ±2.72	103 ±14.6	0.732±0.0728	3.82± 0.301	0.795±0.0854	4.41 ± 0.356
Spleen	1.43 ±0.115	91.2 ±13.3	1.24±0.153	76.7 ± 4.33	0.200±0.0432	17.9 ± 4.61	0.269±0.0515	16.8 ± 2.78
Skeletal muscle	6.70 ±0.568*	1.64± 0.159	6.86±0.342*	1.38± 0.0711	7.46 ±0.906*	1.73± 0.198	8.23 ±1.76*	2.40± 0.101
Blood	1.36 ±0.0599†	4.28± 0.232	1.12±0.0780†	3.04± 0.201	2.63 ±0.318†	8.40± 1.30	2.54 ±0.255†	7.43± 0.607
Feces	4.05 ±0.622	77.1 ± 5.56	2.70±1.22	47.2 ±18.5	12.7 ±1.62	138 ± 16.9	9.81 ±2.03	127 ± 42.2
Urine	1.93 ±0.252	413 ±58.5	2.39±0.716	558 ±97.3	13.5 ±2.22	2230 ±576	9.33 ±1.48	2240 ±235
Placenta	0.293±0.0466	7.96± 0.193			0.200±0.0342	5.26± 0.531		
Fetus (−liver)	1.01 ±0.285	5.84± 0.596			0.333±0.0638	3.64± 0.408		
Fetal liver	0.0741±0.0147	4.44± 0.355			0.0571±0.0173	2.82± 0.230		
Amnionic fluid	0.121±0.0534	15.3 ± 1.89			0.115±0.0399	20.4 ± 9.64		

*Calculated on the basis of dried skeletal muscle comprising 27% of the rat's body weight.
†Calculated on the basis of dried blood comprising 2% of the rat's body weight.

TABLE II

A COMPARISON OF THE RATIOS OF (MEAN DRY WEIGHT OF TISSUE/MEAN LIVE WEIGHT OF RAT) (±S.D.) FOR PREGNANT, NONPREGNANT, AND PREGNANT RATS LESS THE WEIGHT OF THE UTERI AND CONTENTS

Tissue	Twelve nonpregnant rats	Twelve pregnant rats	Twelve pregnant rats less weight of uteri and contents
Spleen	$(0.953 \pm 0.127) \times 10^{-3}$	$(0.752 \pm 0.214) \times 10^{-3}$	$(0.895 \pm 0.230) \times 10^{-3}$
Liver	$(10.3 \pm 0.715) \times 10^{-3}$	$(10.6 \pm 1.43) \times 10^{-3}$	$(12.2 \pm 1.22) \times 10^{-3}$

Table II illustrates another interesting point. When the ratios [weight of dry tissue (g.)/live weight of the rat (g.)] were calculated for the livers and spleens of pregnant and nonpregnant rats, the following comparisons could be made. The ratios for the spleens differed significantly whereas the ratios for the livers did not. However, when the weight of the uterus and contents was subtracted from the live weight of each pregnant rat and the ratios recalculated the reverse was found to be true. That is, the ratios for the spleens did not differ whereas a significant difference existed between the ratios for the livers of the two groups of rats.

Isotope dilution tests performed on the fetal and placental tissue and on the liver of the mother rat yielded the data contained in Table III. The purpose of the isotope dilution tests was to ascertain whether or not the radioactivity present in each tissue was present as unchanged vitamin K_1-C^{14}. Only 12% of the activity in the fetus was present in unchanged form. The placental tissue revealed 59% of the activity to be unchanged vitamin K_1-C^{14}, whereas the value for the maternal liver was 120%. The high value for maternal liver may be due to a sampling error. A value of 78% was previously reported from this laboratory (13) for unchanged vitamin K_1-C^{14} present in the liver of a nonpregnant rat.

TABLE III

ISOTOPE DILUTION TESTS TO DETERMINE THE % OF THE TOTAL RADIOACTIVITY THAT WAS PRESENT AS UNMETABOLIZED VITAMIN K_1-C^{14} IN THE FETAL AND PLACENTAL TISSUES AND IN THE MATERNAL LIVER OF A PREGNANT RAT 24 HOURS AFTER INTRAVENOUS ADMINISTRATION OF VITAMIN K_1-C^{14}

Tissue	% of the injected dose of C^{14} present in the tissue	% of the injected vitamin K_1-C^{14} present in the tissue as K_1-C^{14}	% of the C^{14} present in the tissue which existed as unchanged vitamin K_1-C^{14}
Fetus	1.34	0.164	12
Placenta	0.414	0.293	59
Maternal liver	20.0	24.0	120

Discussion

Dam *et al.* (3) have suggested that the high concentration of vitamin K_1 in the liver and spleen of the rat is related to phagocytosis by the reticulo-endothelial system. The differences in concentration of vitamin K_1-C^{14} and vitamin K_3-C^{14} suggest that either this phagocytosis did not prevent the metabolism of the vitamin or the vitamin K_3-C^{14} suspension was not phagocytozed to as great an extent as the colloidal suspension of vitamin K_1-C^{14}. Radioactivity does pass from the maternal tissues to the fetal tissues and, furthermore, in the case of vitamin K_1-C^{14} a portion of the radioactivity in the fetus exists in unchanged form. The low concentration of vitamin K_1 in fetal and placental tissue probably accounts for the failure of Dam *et al.* to detect it using their extraction procedure and colorimetric estimation (3). Norris and Bennett (7) have presented evidence that the placenta may be a site of prothrombin formation. When vitamin K_1-C^{14} was administered the concentration of radioactivity was significantly greater in the placenta than in either the maternal blood or skeletal muscle. The vitamin K_1-C^{14} equivalent of the placenta was less than that of the maternal liver but significantly greater than the concentration in fetal liver.

Since a ratio may be increased either because the numerator is increased, or the denominator decreased, or both, it is important to note that the increased ratios presented in Table II were due to a marked increase in the weight of the livers of the pregnant rats. The percentage of the injected dose of either vitamin K_1-C^{14} or vitamin K_3-C^{14} present in the livers of the pregnant rats did not differ significantly from that in the livers of nonpregnant rats. It might be suggested that if the liver weight of the pregnant rats had not increased we might have expected a deficit owing to the increased metabolic demands which the uterine contents could present; also the tissues of the pregnant rats were probably hydrated in comparison to the tissues of the nonpregnant rats. If allowance were made for the excess tissue water of the mothers the difference between the ratios would have been even greater than is shown in Table II.

In 1940 Tocantins (14) suggested that the prolonged prothrombin time of the newborn infant was due to a low functional capacity of the liver. Dam (2) pointed out that if this were true then the reduced functional capacity must differ from that observed in chloroform poisoning or cirrhosis as the latter does not respond to vitamin K therapy. Since it has recently been shown (10) that the initial hypoprothrombinemia of the newborn does not respond to vitamin K it would appear that this objection is no longer valid.

Unfortunately, it was not possible to perform isotope dilution tests with vitamin K_3 because the added vitamin K_3 could not be recovered once it was added to the tissue. This observation is supported by other reports (12, 1).

It has been shown in this laboratory that the vitamin K_1 that is excreted in the bile and urine of rats is not present as unchanged vitamin K_1-C^{14} (13). Furthermore, at least two metabolites of vitamin K_3-C^{14} exist as water soluble substances, the diglucuronide, and the monosulphate (5). Our experiments

indicate that the bulk of the radioactivity in the fetus, 88%, exists in some form other than as the unchanged vitamin K_1-C^{14}. We do not know, however, whether the vitamin K_1 crossed the placental barrier in a water soluble form, for example, as an ester, which was then hydrolyzed to vitamin K_1 in the fetus, or whether it was transferred to the fetus as vitamin K_1 and then metabolized to some other derivative.

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DETERMINATION OF Δ^4-3-KETOSTEROIDS BY MEASUREMENT OF THE ABSORBANCE OF THEIR 2,4-DINITROPHENYLHYDRAZONES[1]

W. P. McKinley and W. F. Devlin

Abstract

A rapid, sensitive method suitable for the quantitative determination of ethisterone, methyltestosterone, testosterone, and progesterone is described. The method involves the extraction of the 2,4-dinitrophenylhydrazones of the steroid from the excess dinitrophenylhydrazine reagent with cyclohexane and measurement of the absorbance of the resulting yellow cyclohexane extract. The method has been used in the analysis of a number of tablets and aqueous suspensions containing a mixture of one of these Δ^4-3-ketosteroids and other ingredients.

Introduction

Pharmaceutical preparations which contain either ethisterone, progesterone, methyltestosterone, or testosterone are prepared in the form of capsules, tablets, aqueous suspensions, and oil solutions. Vitamins, phenobarbital, and other chemicals are often present with the steroid in tablet and capsule preparations and most of the available methods for analysis have not dealt with this type of product.

Carol (1) separated mixtures of progesterone and testosterone in aqueous suspension by a chromatographic procedure. The technique was not applied to other types of preparations. Madigan *et al.* (3) obtained a satisfactory recovery of testosterone propionate from oils, by weighing the semicarbazone. However, Umberger (7) obtained high values for testosterone propionate in some sesame oil solutions by this method. In order to overcome this difficulty, Umberger (7) separated mixtures of testosterone propionate and progesterone in oil solutions on a column of Florisil and reported good recoveries. The quantity of each steroid was determined by measuring the absorbance of the isonicotinyl hydrazone. These authors (3, 7) dealt exclusively with oil solutions.

The reagent 2,4-dinitrophenylhydrazine has been used extensively in qualitative and quantitative determinations of progesterone, testosterone, and other steroids for some years. The excess reagent absorbs strongly near the same region as steroid dinitrophenylhydrazones, hence it is advantageous to remove this interfering material.

Reich *et al.* (6) removed the excess 2,4-dinitrophenylhydrazine from the hydrazones of pregnenolone and progesterone by oxidizing it with either Fehling's solution or Benedict's reagent. Gornall and Macdonald (2) described a method of analysis for steroids in which the dinitrophenylhydrazone of the steroid was converted to a form which gave a red-colored

[1]Manuscript received February 26, 1957.
Contribution from Department of National Health and Welfare, Food and Drug Laboratory, Ottawa, Ontario.

solution, by the addition of 4 *N* NaOH. The addition of alkali also brings about a change in the dinitrophenylhydrazine, resulting in a reddish-amber-colored "blank". This procedure was utilized by these workers in an extensive study of steroids in biological materials but not for pharmaceutical mixtures. Since Gornall and Macdonald (2) were more interested in adrenal steroids, only a brief reference was made to the dinitrophenylhydrazones of progesterone and testosterone propionate.

In this paper a procedure is described for the quantitative determination of the dinitrophenylhydrazones of methyltestosterone, testosterone, ethisterone, and progesterone free from the excess reagent.

Experimental

Reagents

1. 2,4-Dinitrophenylhydrazine (DNPH): the reagent is the same as that used by Gornall and Macdonald (4), consisting of a solution of 1 mg. of British Drug House, reagent grade DNPH per ml. of solvent. The solvent consists of 1 volume of hydrochloric acid (sp. gr. 1.19) to 3 volumes of methanol. The reagent was made up fresh each day.
2. Stock solution: 100 mg. of crystalline steroid (U.S.P.) which had been dried over phosphorus pentoxide to a constant weight was dissolved in 60 ml. of absolute methanol in a 100 ml. volumetric flask and made to volume with methanol. The solution was stored in the refrigerator, away from direct light. A working solution was prepared by diluting 10 ml. of the stock solution to 100 ml. with methanol.
3. Methanol: Merck reagent grade methanol was satisfactory without further purification. If the alcohol contains an appreciable amount of aldehydes or ketones, the cyclohexane "blank" will be colored.
4. Cyclohexane (hexa-hydro-benzene): B.D.H. reagent grade was used without further purification.
5. Sodium hydroxide: approximately 4 *N* NaOH.
6. Petroleum ether: analytical reagent grade, boiling range 30°–75° C.

Elimination of Excess Reagent (DNPH)

The 2,4-dinitrophenylhydrazine used in the reaction has an absorbance maximum at 345 mμ, which is near the absorbance maximum for most of the steroid dinitrophenylhydrazones. Several combinations of solvent systems were examined, in an attempt to fractionate the excess reagent from the dinitrophenylhydrazones.

Cyclohexane or chloroform extracts the dinitrophenylhydrazones from a reaction mixture consisting of a saturated solution of DNPH in 2 *N* HCl or 30% acetic acid, but a portion of the DNPH reagent is also removed. The portion of DNPH reagent which is removed can be re-extracted into 33% sulphuric acid from the cyclohexane but not from chloroform. This procedure allows one to obtain a linear relationship between concentration and absorbance of the cyclohexane extract but since the technique involves two extractions, it was decided to try other systems. Several organic bases, such as

diethanolamine, triethanolamine, isopropylamine, and tetramethyl ammonium hydroxide were tried, as solvents which would prevent the extraction of the DNPH reagent, but none was entirely satisfactory.

Gornall and Macdonald (2) stated that a good deal of absorption occurred, at times, below 400 mμ after the dinitrophenylhydrazones had been converted to the red color by the addition of 4 *N* NaOH. It seemed possible that this absorption may have been due to incomplete conversion of all the molecular species which absorb below 400 mμ to those which absorb in the range between 440 and 495 mμ after the addition of the alkali.

The Gornall and Macdonald (2) procedure was used to prepare the dinitrophenylhydrazone of methyltestosterone in varying concentrations. Six milliliters of cyclohexane was added to each preparation containing the dinitrophenylhydrazone and also to a reagent "blank". The cyclohexane extract of the reagent "blank" was colorless, while the extracts of the preparations containing the dinitrophenylhydrazone appeared as a clear, yellow solution. The lower alkaline alcoholic layer remained red. Dilution of this lower layer with water resulted in a disappearance of the red color and the appearance of a deeper yellow in the upper cyclohexane layer, with no additional color in the cyclohexane extract of the reagent "blank". Therefore, the addition of alkali had altered the DNPH reagent in such a manner that it was no longer extractable with cyclohexane after the addition of water, while the dinitrophenylhydrazones were extractable with this solvent.

Chloroform was discarded as a suitable solvent since it continued to extract a portion of the reagent from this reaction mixture.

The Effect of Alcohol Concentration on the Development of the Red Color

In order to substantiate the observation mentioned in the preceding paragraph concerning the disappearance of the red color after dilution of the alkaline alcoholic phase, the following test was carried out.

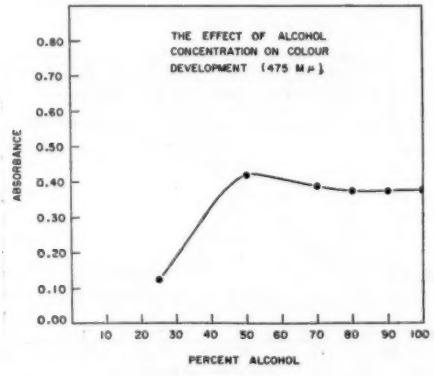

FIG. 1. A graph illustrating the effect of mixtures of methanol and water of varying proportions on the red color formed, after the addition of 4 *N* NaOH.

Forty-five micrograms of testosterone in 0.5 ml. of methanol was added to each cylinder of a series of twelve. The reaction was carried out as described for the method. After the addition of the NaOH to form the red color, 10 ml. of methanol was added to each cylinder. The concentration of the added methanol varied as shown in Fig. 1. It is noted that the absorbance of the red color (475 mμ) begins to disappear rapidly as the concentration of the methanol is reduced below 50%.

This observation illustrates the necessity of using high concentrations of alcohol for maximum color development not only for solutions but also for the detection of dinitrophenylhydrazones of ketosteroids on paper chromatograms. It may be mentioned that isopropylamine or tetramethylammonium hydroxide are both capable of bringing about this change from a yellow to a red-colored dinitrophenylhydrazone.

Extraction of the Dinitrophenylhydrazones with Cyclohexane

In order to determine the optimum conditions for the extraction of the dinitrophenylhydrazones with cyclohexane, the following test was conducted. A series of cylinders, each containing 0.5 ml. of a methanolic solution of testosterone, were prepared. The reaction was carried out as described in the method to the point where the sodium hydroxide is added. The addition of the sodium hydroxide was followed by 10 ml. of aqueous methanol to each cylinder to give a final concentration of methanol ranging from 30 to 80% as shown in Fig. 2. After the addition of the aqueous methanol, 6 ml. of cyclohexane was added to each cylinder and the mixture was shaken. The absorbance of each of the cyclohexane extracts was plotted against the alcohol concentration. This procedure was repeated for progesterone and the data for the extraction of the two dinitrophenylhydrazones has been presented in graphical form in Fig. 2. It may be seen from Fig. 2 that the extraction of the dinitrophenylhydrazones is maximal when the concentration of the methanol in the alkaline solution is less than 50%, the concentration at which the red color begins to disappear (Fig. 1). The concentration of sodium hydroxide in the final volume is approximately 0.04 *N*.

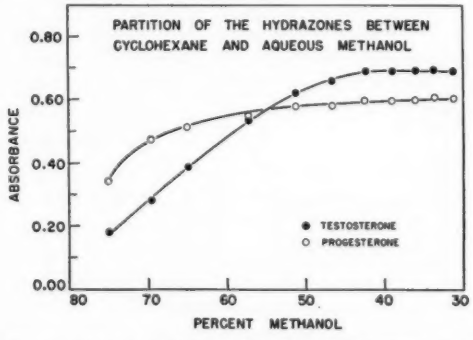

FIG. 2. Absorbance values for the dinitrophenylhydrazones of progesterone and testosterone extracted by cyclohexane from varying strengths of aqueous methanol.

Reaction Time

The time required to obtain an optimum amount of product was determined by employing the same amount of steroid and DNPH reagent in each cylinder, but allowing the reaction to proceed for varying lengths of time before the addition of alkali. The absorbance of the cyclohexane extract at 375 mμ was plotted for the dinitrophenylhydrazones of progesterone and testosterone against the time of reaction as shown in Fig. 3. The maximum amount of dinitrophenylhydrazone of testosterone is formed in 5 minutes. The maximum amount of cyclohexane-extractable dinitrophenylhydrazone of progesterone is formed in 3 minutes and begins to decrease if the reaction is allowed to proceed for more than 10 minutes. This decrease in cyclohexane-extractable material coincides with the appearance of a precipitate in the reaction cylinder. It is believed that the cyclohexane-extractable material may be the monohydrazone while the precipitate is probably the bisdinitrophenylhydrazone, the form produced when the gravimetric procedure (U.S.P. XV) is employed.

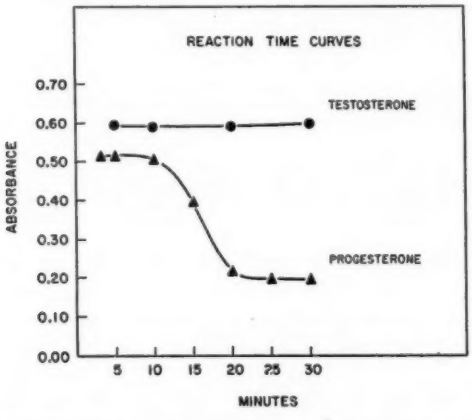

FIG. 3. Graphs illustrating the absorbance of the cyclohexane extract of the dinitrophenylhydrazones of testosterone and progesterone from reaction mixtures which had been allowed to react for different lengths of time. The dinitrophenylhydrazones of methyltestosterone and testosterone propionate behave in a manner similar to that shown for the dinitrophenylhydrazone of testosterone.

Absorbance Maxima

Cyclohexane extracts of several steroid hydrazones were prepared and the absorbance curves were recorded with a Cary recording spectrophotometer. The absorbance peaks are presented in Table I and the curves for four of these are shown in Fig. 4. The absorbance maxima are all very similar and near the absorbance maximum for the DNPH reagent. This indicates the necessity of removing the excess reagent before a measurement of the dinitrophenylhydrazone is made.

TABLE I

ABSORBANCE PEAKS OF KETO STEROID DINITROPHENYLHYDRAZONES*

Compound	Max. peak (mμ)	Minor peak (mμ)	Position of functional groups		
			Keto group	Phenolic OH	Alcoholic OH
Androsterone	350	425	17	—	3
Pregnenolone acetate	350	425	20	3	—
Oestrone	363	425	17	3	—
Progesterone	363	(398) (440)	20, 3	—	—
Testosterone	375	440	3	—	17
Methyl testosterone	375	440	3	—	17
Testosterone propionate	375	440	3	—	—

*The dinitrophenylhydrazones of androsterone, pregnenolone acetate, and oestrone were prepared at 59° C. rather than at room temperature, and the completion of extraction of these dinitrophenylhydrazones was not studied.

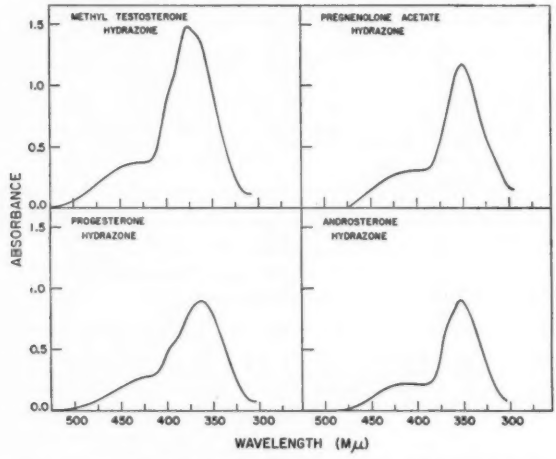

FIG. 4. Absorbance curves of dinitrophenylhydrazones of steroids with keto groups at carbons 3, 17, and 20 as shown in Table I.

Relationship between Absorbance and Steroid Concentration

The relationship between absorbance and quantity of dinitrophenylhydrazone is illustrated in Fig. 5. The lower line (solid circles) represents the relationship between absorbance and quantity of testosterone dinitrophenylhydrazone in micrograms of steroid per final volume of solution. This relationship represents absorbance of the red color in the alkaline, alcoholic solution measured at 475 mμ by the Gornall and Macdonald (2) procedure. The line represented by hollow circles is the relationship between absorbance of the cyclohexane extract at 375 mμ and the amount of steroid per final volume of extract. The amounts of testosterone and the final volumes were

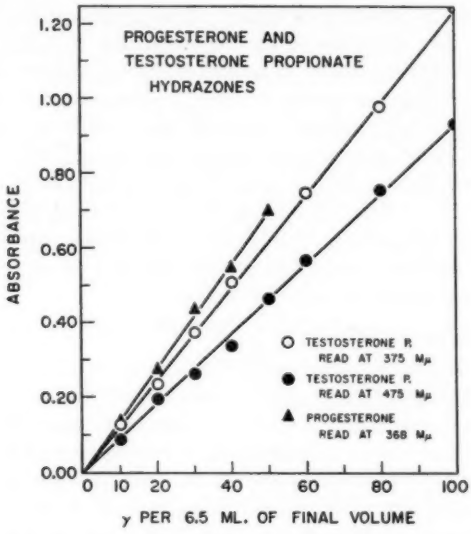

FIG. 5. The plots show the relationship between absorbance and quantity of dinitrophenylhydrazone in a final volume of 6.5 ml. in each instance. The γ per 6.5 ml. refers to the quantity in micrograms of the testosterone propionate or progesterone present in the final volume as the hydrazone.

A comparison is made of the absorbance of the dinitrophenylhydrazones of testosterone propionate at 375 mμ (yellow) and at 475 mμ (red, after addition of sodium hydroxide). The use of 6.5 ml. of cyclohexane gave the same final concentrations for the testosterone propionate hydrazone.

the same for the two wavelengths. The measurements at 375 mμ are somewhat more sensitive and in our experience more readily reproduced. The line through the points marked as triangles shows the relationship between absorbance and quantity per final volume of solution of the progesterone dinitrophenylhydrazone measured at 368 mμ.

Method

To a series of glass-stoppered 25 ml. cylinders add 0.0, 0.1, 0.2, 0.3, 0.4, and 0.5 ml. of the working standard steroid solution. To each of the cylinders containing the standard, add sufficient methanol to make the volume to 0.5 ml. If the sample to be analyzed contains less than 100 micrograms per ml., or if the sample is dissolved in another solvent, one may remove the solvent with a slow stream of nitrogen. The 0.5 ml. of methanol is then added in such a manner that all the steroid which may be adhering to the side of the cylinder is washed from the glass surface. After the addition of the alcohol, 0.5 ml. of the reagent is added and the reaction time is checked by means of a stop watch. The reaction time is the period from the addition of the reagent to the addition of the sodium hydroxide.

The reaction time for ethisterone and the testosterones is 10 minutes and for progesterone it is 3 minutes. The reaction time for progesterone is very

critical and should be checked carefully. Each cylinder should be given one brief shake after the addition of the reagent. At the end of the reaction period, 0.5 ml. of 4.0 *N* NaOH is added and the cylinder is shaken. The addition of the alkali prevents the reaction of progesterone from proceeding further. The cylinders are allowed to stand for 5 minutes and then 6.0 ml. of cyclohexane and 10 ml. of water are added to each cylinder. The cylinders are shaken vigorously for 1 minute and, after they have been allowed to stand for 15 minutes, 3 ml. of the cyclohexane layer is removed by a pipette and the absorbance is read against the cyclohexane extract from the reagent "blank". The absorbance readings for progesterone are taken at 368 mμ and for ethisterone and the testosterones at 375 mμ.

Sampling of Pharmaceuticals

Tablets

A minimum of five weighed tablets are reduced to a fine powder without appreciable loss. A portion of the powdered tablets containing approximately 10 mg. of the steroid is weighed accurately. The weighed quantity of powder is placed into a 100 ml. glass-stoppered flask and, after the addition of 80 ml. of hot methanol, the flask is placed on a mechanical shaker for 1 hour. The extract is filtered through Whatman No. 1 filter paper and the filter paper is washed with sufficient methanol to make the volume up to 100 ml. of solution. The concentration is such that 0.5 ml. contains approximately 50 micrograms of steroid.

Aqueous Suspensions

The aqueous suspensions must be shaken vigorously just before the sample is taken, in order to obtain uniform distribution of the precipitated material. An amount of suspension containing approximately 25 mg. of steroid is measured with a 1 cc. serological pipette and the pipette is washed with methanol. The sample and washings are placed into a 250 ml. volumetric flask, to which is added 200 ml. of hot methanol. The flask is stoppered, shaken, the contents filtered, and made to volume in the manner described for the tablets. This procedure will give a final concentration of approximately 100 micrograms per ml.

Ethyl Oleate Solutions

A weighed sample containing 25 mg. of the steroid is dissolved in 40 ml. of petroleum ether (boiling range 30°–75° C.) which has been saturated with 90% ethanol. The petroleum ether solution is extracted with eight 15 ml. portions of 90% ethanol which has been saturated with petroleum ether (2). The combined alcohol extracts are evaporated to dryness. The residue is dissolved in methanol, transferred to a 250 ml. volumetric flask, and made to volume with methanol.

Special Precautions

Some tablets and capsules on the market contain colored components which may be extracted and end up in the cyclohexane layer. In such a case a

colored "blank" is prepared by adding the reagents in the regular way, with the important exception that the 4.0 *N* NaOH is added before the alcoholic solution containing the same amount of steroid as the sample.

This step allows the colored material to be measured but the steroid hydrazone product is not formed when the reagents are added in this order. This "blank" is read against the normal reagent "blank" and the reading is substracted from the reading for the sample to be analyzed. For example:

A = reading of unknown against reagent "blank";

B = reading of the colored "blank" against the reagent "blank".

True reading for the unknown is then the difference between A and B.

Results and Discussions

Pharmaceutical preparations of methyltestosterone tablets were analyzed by the proposed method and by the U.S.P. XV gravimetric procedure for testosterone propionate injection, in which the methyltestosterone was weighed as the semicarbazone. The results shown in Table II agree quite favorably for three of the four samples, but the fourth sample gave a value which was 21.8% higher than the labelled claim for the gravimetric method. An aqueous extract of these tablets reduced Benedict's reagent and formed a dinitrophenylhydrazone which precipitated from the reaction medium. The

TABLE II

COMPARISON OF METHODS FOR THE ANALYSIS OF COMMERCIAL PREPARATIONS OF METHYLTESTOSTERONE

Sample No.	Type of preparation	Labelled conc.	Proposed method (%)	U.S.P. XV method (%)
1	Tablets	10 mg./tab.	95.6	97.8
2	"	10 " "	99.2	96.0
3	"	10 " "	94.2	95.8
4	"	10 " "	89.3	121.8

solution containing this dinitrophenylhydrazone turned red upon the addition of sodium hydroxide and remained red after dilution with water. The dinitrophenylhydrazone was not extracted with cyclohexane, which explains why it did not give a high value when analyzed by this procedure. However, the material in the aqueous extract did react readily with semicarbazide to form a precipitate and this explains the interference when the extract was analyzed by the gravimetric procedure. Table III has been included to illustrate the recovery values obtained when samples containing methyltestosterone or testosterone prepared in a number of different pharmaceutical forms were analyzed by the proposed method. Results are presented in Table IV for pharmaceutical preparations of progesterone, which were analyzed by the proposed method, the U.S.P. XV gravimetric procedure for progesterone injections, and the U.S.P. XV gravimetric method for aqueous suspensions. The U.S.P. XV procedure for progesterone injection involves the formation of

TABLE III

ANALYSIS OF PHARMACEUTICAL PREPARATIONS OF METHYLTESTOSTERONE

Sample No.	Type of preparation	Labelled conc.	Found (%)
1	Tablet	10 mg./tab.	95.6
2	"	10 "	96.6
3	"	10 "	88.6
4	"	10 "	90.0
5	"	10 "	94.2
6	"	10 "	99.2
7	"	10 "	89.3
8	"	3 mg./tab.	91.0
9*	Aqueous suspension	25 mg./cc.	93.6

*Sample 9 was a testosterone sample.

bisdinitrophenylhydrazone of progesterone under reflux conditions. The precipitated bisdinitrophenylhydrazone is washed to remove the excess DNPH reagent, dried, and weighed. The U.S.P. XV method for progesterone in aqueous suspension calls for filtering a portion of the sample, washing the precipitate, and weighing the dried residue. The U.S.P. XV procedure for progesterone injection agreed well with the proposed method for all the samples analyzed, but the U.S.P. XV method for aqueous suspension gave high values for most of the samples tested.

TABLE IV

COMPARISON OF METHODS OF ANALYSIS OF COMMERCIAL PREPARATIONS OF PROGESTERONE

Sample No.	Type of preparation	Labelled conc.	Found		
			Proposed method (%)	U.S.P. XV* (%)	U.S.P. XV† (%)
1	Ethyloleate	5 mg./cc.	97.4	97.2	—
2	Vaginal tablets	25 mg./tab.	93.8	96.0	—
3	Aqueous susp.	25 mg./cc.	75.0	76.6	102.4
4	" "	25 "	78.6	79.1	100.4
5	" "	25 "	107.0	104.1	118.0
6	" "	25 "	101.2	103.6	103.6

*Refers to the U.S.P. XV method for sterile progesterone injection.
†Refers to the U.S.P. XV method for sterile progesterone suspension.

The data in Table V demonstrated that the proposed method may be used in the analysis of pharmaceutical preparations containing ethisterone. The recoveries in most cases were within the limits allowed. This technique has not been compared with other methods since there is no official method of analysis for ethisterone at present.

TABLE V

ANALYSIS OF PHARMACEUTICAL PREPARATIONS OF ETHISTERONE (ANHYDROHYDROXY PROGESTERONE) BY THE PROPOSED METHOD

Sample No.	Type of preparation	Labelled conc.	Found (%)
1	Tablets	5 mg./tab.	96.0
2	"	5 " "	92.4
3	"	10 " "	94.2
4	"	10 " "	90.8
5	"	10 " "	92.2
6	"	10 " "	84.0

The average absorbance and standard deviation for each compound were calculated for four separate determinations at each of the 10 and 50 microgram levels for each of the four steroids listed in Table VI. The standard deviation is of the same order at the two levels for a given compound, except for progesterone, the reaction time of which must be very carefully controlled.

TABLE VI

PRECISION OF METHOD FOR DETERMINATIONS OF THE COMPOUNDS TESTOSTERONE, METHYLTESTOSTERONE, ETHISTERONE, AND PROGESTERONE AT TWO LEVELS (WHERE THE 10 AND 50γ LEVELS REFER TO MICROGRAMS OF STEROID IN THE FINAL 6.5 ML. VOLUME OF CYCLOHEXANE)

	10γ level		50γ level	
	Average absorbance (4 determinations)	Standard deviation	Average absorbance (4 determinations)	Standard deviation
Testosterone	0.144	± 0.004	0.779	± 0.005
Methyl testosterone	0.143	± 0.006	0.740	± 0.006
Ethisterone	0.131	± 0.002	0.667	± 0.003
Progesterone	0.127	± 0.004	0.678	± 0.009

The proposed method has been used to analyze a number of pharmaceutical products containing a variety of substances, such as estrogenic materials, chlorobutanol, procaine, pectin, sodium acetate, merthiolate, phenobarbital, thiosalicylates, vitamins, salts, and other components in addition to either testosterone, methyltestosterone, ethisterone, or progesterone. This method gives good recoveries for most of the pharmaceutical preparations and compares favorably with the hydrazone methods described in the U.S.P. XV. This method is more specific than the U.S.P. procedure because it may be used to analyze samples containing keto or aldehydic sugars and organic acids such as pyruvic and opianic. It is sensitive and much less laborious than most of the existing pharmacopoeial procedures. It may be used for the analysis of a large variety of commercial preparations containing either progesterone, testosterone, methyltestosterone, or ethisterone when chromatographic (5) and electrophoretic (4) techniques are used for the identification of the component.

Acknowledgments

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A ROUTINE PROCEDURE FOR THE DETERMINATION OF CATECHOLAMINES IN URINE AND TISSUES[1]

THEODORE L. SOURKES AND BORIS D. DRUJAN

Abstract

A simplified procedure for the determination of catecholamines, suitable for routine laboratory use, has been developed. The whole of the adsorption–elution process, by which the catecholamines are concentrated and purified, is performed in one flask. The potentiometric titration required at this stage in the adsorption of the catecholamines on alumina is avoided by the use of phenolphthalein as an internal indicator. The fluorimetric estimation in the second stage of the procedure is based upon lutin formation, a reaction nearly specific for adrenaline and noradrenaline. The small error resulting from the presence of hydroxytyramine in the urine is discussed. The method provides for the determination of losses encountered in the extraction process, so that each run includes an internal correction rather than an assumed average factor. Examples of the utility of the method are given. These include the determination of the rate of excretion of catecholamines in the urine, the effect of insulin on this rate, and the estimation of the catecholamine content of rat organs.

Introduction

In the last few years sensitive methods have been developed for the determination of adrenaline and noradrenaline in body fluids and organs. The availability of these methods has logically been reflected in their use in clinical diagnosis, particularly in the differential diagnosis of pheochromocytoma, as well as in research on the physiology of the autonomic nervous system. Unfortunately, the time-consuming nature of the procedures currently available is an obstacle to their wider utilization in both clinical and research laboratories. It should be emphasized that of the numerous steps involved in the determination of catecholamines none is particularly different from ordinary laboratory practices. However, all of them in conjunction demand considerable time and attention on the part of the chemist and are therefore not so readily adaptable to routine.

The procedure to be described was developed to facilitate the handling of relatively large numbers of urine and tissue samples and, in the practical work of research here, it has been found to avoid many of the drawbacks mentioned above. Thus, it is possible for one technician to process as many as 20 samples (e.g. eight urines in duplicate, plus controls) in less than the usual working day. It is this simplification of the existing extraction methods and choice of suitable conditions for fluorescence development which may recommend the present procedure for routine use, especially in the clinical laboratory.

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Review of Methods

The procedures for estimating catecholamines (CA) all entail a preliminary purification step in which the catechols are selectively adsorbed on alumina gel (10, 29) or on aluminum oxide suitably prepared for chromatography (14, 23, 39). After the alumina has been washed, the catechols are eluted with dilute acetic acid and are then ready for determination. Catechol derivatives found in urine are adrenaline, noradrenaline, 3-hydroxytyramine ("dopamine") (9, 17, 18), and 3,4-dihydroxyphenylacetic acid (catecholacetic acid, "dopacetic" acid) (5, 36). Dihydroxyphenylalanine (DOPA) has been detected in extracts of heart and of adrenal gland (16); and both DOPA and 3-hydroxytyramine have been identified in tumor tissue from a case of pheochromocytoma (37). The latter compound has also been found in nerve extracts (28). Of these substances, the physiologically significant ones are adrenaline and noradrenaline. They are the catecholamines determined by the usual biological assay methods. Small amounts of isopropylnoradrenaline have been reported to occur in adrenal extracts (22) but this has not yet been confirmed. Adrenalone, the keto analogue of adrenaline, has been claimed as a product of the action of blood enzymes on adrenaline (20, 31).

Three characteristic reactions of adrenaline and noradrenaline have been used in their chemical estimation.

(*1*) *Chrome formation.*—The CA in the eluate are oxidized to form a mixture of adrenochrome and noradrenochrome. The oxidant usually used for this is iodine (3, 8) so that some of the corresponding iodochromes are formed also. Adrenochrome is the orthoquinone of N-methyl-3,5,6-trihydroxy-2,3-dihydroindole. As the name implies, the chromes are colored; their concentration can be measured in a colorimeter at 529 mμ. In our experience the least amounts measurable are 1–2 μg., so that this method has a limited usefulness.

(*2*) *Lutin formation.*—Adrenolutin is N-methyl-3,5,6-trihydroxyindole. Lutins are formed by the alkaline rearrangement of the "chromes", of which they are actually isomers. They are unstable, but can be protected temporarily by ascorbic acid (2, 15, 24), permitting measurement of their fluorescence. Oxidants which have been used to convert adrenaline and noradrenaline to the corresponding lutins are ferricyanide (3, 7), manganese dioxide (24), and iodine (34). As little as 0.01 μg. of adrenaline can be measured in this way.

(*3*) *Ethylenediamine condensation.*—Under alkaline conditions catechol derivatives condense with ethylenediamine to form fluorescent substances (26). This is the most sensitive reaction known for detecting CA and, as developed by Weil-Malherbe and Bone (39), has been used for the quantitative estimation of adrenaline and noradrenaline in plasma (35) and other tissues (25). It is useful for detecting as little as 0.002 μg. of CA. Townsend has found (32, 33) average concentrations of adrenaline and noradrenaline in normal plasma of the same order as those found by the authors of the ethylenediamine method (39), but the large associated standard error has limited application of the procedure in this laboratory. Burn and Field (1)

have used the ethylenediamine reaction to detect the abnormally high concentration of CA in the urine of cases of pheochromocytoma. The urinary extracts give a color with the reagent.

In order to determine adrenaline and noradrenaline separately, two techniques have been used. In the first, the differences in the emission spectrum of the fluorescent products have been utilized by measuring fluorescence successively with two selected secondary filters (39). The second technique makes use of the greater rate of oxidation of adrenaline under selected reaction conditions, as at pH's below 6; duplicate aliquots of unknowns are adjusted to pH 4 and pH 6, respectively, and the ultimate fluorescence values obtained are substituted in a pair of simultaneous equations (7, 8). Neither the first (21, 32) nor the second technique ((15), and the authors' unpublished work) has been found satisfactory. The "differential pH method" yields a variable error for different ratios of adrenaline and noradrenaline (12).

Against the problem of separate quantitative identification of the two chief catecholamines, the need for which is unquestioned, there are many instances where a figure for total catecholamines is satisfactory. This is the case where urinary catecholamines are estimated as an aid in the differential diagnosis of pheochromocytoma. In animal tissues, Euler has shown (6) that the CA content is almost entirely noradrenaline in spleen, liver, nerve trunks, brain, spinal cord, and striated muscle; in the salivary glands, heart, and kidney, adrenaline constitutes a significant portion of the total. Furthermore, most of the CA content of the adrenal glands of rat, hamster, guinea pig, and rabbit, four common laboratory animals, consists of adrenaline (6, 19, 40) so that for some types of experiment any major change in CA level would presumably involve this compound. It is important, for comparison's sake, to note that results obtained by biological assay are subject to a large variation; the standard error is commonly between 10 and 30% (6).

Clearly what is needed for differentiation of the two sympathetic neurohumors are reactions specific for each of them. The techniques used at present for this differentiation, including the methods of biological assay, are only approximations to ideal conditions and introduce their own errors.

Method

1. Extraction of Catechols

A. From Urine

Urine is collected in acid (2 ml. concentrated hydrochloric acid diluted with 2 ml. water, and containing a few drops of toluene) and is filtered if not clear. Aliquots of 100 ml. or, alternatively, 10% of the 24 hour urine volume are measured into a 250 ml. Pyrex centrifuge bottle containing 10 ml. of 10% Versene and one drop of 1% phenolphthalein. Two grams of neutral ("non-alkaline") or acid-washed alumina of chromatographic grade are added. The solution is neutralized by the careful addition of 20% sodium hydroxide, with constant swirling of the suspension. As the phenolphthalein end point is

approached, 4% sodium hydroxide (= 1 normal) is used. The pink indicator color is not immediately stable since acid is slowly released from the alumina. The bottle is shaken vigorously (mechanical agitator) for 5 minutes. More alkali is then added, if necessary, to restore the indicator color and the 5 minute shaking is repeated. Usually the pink color is permanent at this time.

The alumina is allowed to settle or is spun down in the centrifuge, and the supernatant is removed by means of suction (water pump) applied through a fine-bore pipette. To the moist alumina, 10 ml. of 0.2 *M* sodium acetate, previously adjusted to about pH 8.3 (phenolphthalein) are added. The suspension is again shaken for 5 minutes and the supernatant is discarded (suction) as before. Ten milliliters of glass-distilled water is added to the alumina, and the above procedure of shaking and removal of the supernatant is repeated. For the elution, 10 ml. of 0.5 *M* acetic acid is shaken with the alumina for 10 minutes, at the end of which time the supernatant is decanted into a test tube, centrifuged, and preserved for the CA estimation. The procedure may be conveniently interrupted at this stage; if so, the eluates should be stoppered and held in the refrigerator until required.

B. From Tissues

The tissue is weighed and ground in a blendor with 10 times its weight of 2% trichloracetic acid (TCA). For small organs, such as rat adrenals, the VirTis "45" homogenizer has been found very useful. A pair of glands is ground with 2 ml. of TCA for 1 minute at the highest speed, and the extract is made to 50 ml. with 10% TCA. Suitable aliquots, e.g. 10–20 ml., are taken for treatment exactly as described for urine.

2. Fluorimetry

A. Reagents

(*a*) Iodine solution, 0.004 *N*, diluted daily, stepwise, from 0.1 *N*.

(*b*) Sodium thiosulphate solution, 0.005 *N*.

(*c*) Sodium hydroxide – ascorbic acid solution. Dissolve 20 mg. of ascorbic acid in 1 ml. water; add 9 ml. of 20% NaOH; mix well. The solution is stable for a few hours.

(*d*) Sodium acetate buffer, 1 *M*, pH 6.0.

(*e*) Noradrenaline stock solution. Make up 12.0 mg. of noradrenaline hydrochloride (Winthrop-Stearns Ltd.) to 100 ml. with 0.2 *N* acetic acid. Store in the refrigerator. This solution is equivalent to 100 μg. of noradrenaline base per ml.

(*f*) Noradrenaline working solution: Dilute 1.0 ml. of stock solution to 100 ml. with 0.02 *N* acetic acid; equivalent to 1.0 μg. per ml. Prepare fresh daily. Store in dark glass bottle, in the cold.

B. Lutin Formation

Two aliquots, 0.2 and 0.4 ml., respectively, of each eluate are used. These are made up to 1.0 ml. with water and then 2.0 ml. of the buffer, pH 6.0, is pipetted in. For the oxidation 0.3 ml. of the iodine solution is added. At the end of exactly 3 minutes, 0.3 ml. of the thiosulphate solution is added with

shaking, to despatch the excess iodine. One milliliter of the sodium hydroxide – ascorbic acid solution is added to isomerize the chromes to the lutins and to stabilize the latter (7). At the same time as the eluates are run a standard curve is prepared, using 0.05, 0.10, 0.20, and 0.30 μg. of noradrenaline. The reagent blank consists of all the ingredients except noradrenaline. These tubes are all treated in the same fashion as the "unknowns".

In this laboratory fluorescence of the solution is measured with the Farrand Model A Fluorometer, using the following filters: primary, Corning 5113 (blue) and 3389 (pale yellow) selecting the 436 mμ line of the mercury vapor lamp source; secondary, Corning 3486 (yellow). Other yellow filters have been tried in the secondary position, but the one mentioned yields standard fluorescence–concentration curves meeting the requirements of linearity better than the others. The largest opening (No. 1) is used between light source and primary filter combination. The galvanometer is adjusted to 100 mm. deflection using in the "Standard" cuvette position a solution of quinine sulphate or sodium fluorescein whose fluorescence approximately matches that of 0.30 μg. of noradrenaline carried to the lutin stage. Other styles of fluorometer on the market are undoubtedly adaptable in a similar way.

3. Calculations

All galvanometer readings are corrected for the deflection observed with the reagent blank. From the standard curve an average factor, F, is calculated as F mm. scale length deflection per μg. of noradrenaline. If the volume of the test solution is E ml. (10 ml. of eluate in this procedure) and the aliquot taken is A ml. (0.2 or 0.4 ml.), then the total catecholamines, estimated as noradrenaline, is $(R/F) \times (E/A)$ μg., where R is the galvanometer reading in millimeters. The average CA content estimated using the two aliquots is taken as the result.

Since recovery of added CA is not complete, a suitable correction must be applied to the figure calculated above. This is obtained by treating four bottles of urine (or tissue extract) having known amounts of CA (e.g. duplicates of samples to be determined in the run) with given amounts of noradrenaline; 2, 4, 6, and 10 μg., respectively, may be used. These samples are treated exactly as the others, and the amount of CA present is determined. A line is fitted to the points plotted as "μg. of noradrenaline added" versus "μg. CA recovered"; and an equation of the form:

$$\text{noradrenaline added} = A + B\ (\text{CA found})$$

is derived. Usually the line passes through the point (0, 0), in which case $A = 0$. All unknowns are corrected by the use of this equation.

Alternatively, the corrected galvanometer readings may be plotted against the amount of noradrenaline added to the samples, and the line of best fit to these points used to convert the readings for the unknowns directly to "CA found, estimated as noradrenaline".

TABLE I

RECOVERY OF ADDED NORADRENALINE FROM URINE AND RAT ADRENAL GLAND EXTRACTS

	% recovery							
	15–24	25–34	35–44	45–54	55–64	65–74	75–84	85–94
	No. determinations							
Urine*	3	3	7	2	4	4	0	1
Adrenals†	1			6	4	1		

*24 determinations, each based on two to four concentrations of noradrenaline.
†12 determinations, each based on four concentrations of noradrenaline.

4. Recovery of Added Noradrenaline

Recovery is variable between runs, as shown in Table I, but with strict standardization of the conditions of extraction it has usually been above 35%. The recovery from TCA extracts of rat adrenals is better, about 50–60%.

Results and Discussion

Relative Fluorescence of Catecholamines

The fluorescences of several catecholamines and related compounds are shown in Table II. It is evident that adrenaline and noradrenaline exhibit much more fluorescence than the other substances listed.

TABLE II

RELATIVE INTENSITY OF FLUORESCENCE OF CATECHOLAMINES

Compound	Range of conc. studied	Relative fluorescence
l-Noradrenaline	0.1 – 0.4	100
l-Adrenaline	0.05– 0.40	145
dl-Isuprel	0.02– 0.40	82
3-Hydroxytyramine ("Dopamine")	1–10	4
DL-Adrenalone*	2–20	0
DL-Noradrenalone* (arterenone)	2–20	0
DL-Dihydroxyphenylalanine (DOPA)	2–28	0

*Supplied by Sterling-Winthrop Research Institute. These compounds showed some fluorescence at the 50 μg. level.

Rate of Excretion of Catecholamines in Urine

In 41 consecutive determinations on 19 subjects the mean rate of excretion of CA in a 2–4 hour period after waking (variously within the 7.00–11.30 A.M. span) was 47.4 ng./minute with a standard error of 6.3 (1 ng. (nanogram) $= 10^{-9}$ g.). This may be reasonably compared with the data provided by Euler and his colleagues (11) in their report on diurnal variation in the excretion of adrenaline and noradrenaline in healthy young adults. In the morning urine (8–11 A.M.) there were about 5 and 28 ng./minute, respectively, of these compounds as determined by biological assay. In order to make the

comparison between Euler's estimate and the present chemical determination of CA, as noradrenaline, several factors must be taken into account. First of all, under the conditions of oxidation used here, adrenaline yields almost 1.5 times as much fluorescence as an equivalent amount of noradrenaline (Table II). Secondly, the Swedish data must be corrected for about 20% loss of CA in the extraction (11). Thirdly, the chemical, but not the biological, determination detects 3-hydroxytyramine; this compound occurs in urine to the extent of 0.1–0.2 mg./24 hours (9), i.e. in about 3- to 7-fold the concentration of noradrenaline. However, it fluoresces very much less, about 4% of the noradrenaline figure under the present oxidative conditions. Pitkanen (27) has had a similar result. When these factors are considered, Euler's average rate becomes 47–50 ng. of CA (as noradrenaline) per minute, with which the present estimate is in excellent agreement.

Effect of Insulin

The influence of insulin on the rate of excretion of CA has been investigated in seven patients receiving a total of 16 administrations of 30–70 I.U. of insulin. In the 4 hours between arising and the administration of insulin the rate of excretion was 38.1 ng./minute, whereas during the period of somnolence induced by insulin the rate rose to 103.3 ng./minute (also over a 4 hour period). The standard error of this difference is $\pm$ 10.0 ng./minute, so that the effect of insulin is highly significant ($t = 6.52$, $P < 1\%$). Euler and Luft (13) found that the intravenous injection of 0.1 I.U. of insulin per kg. body weight resulted in a 10-fold increase in adrenaline excretion rate, but without a change in the noradrenaline output. Elmadjian *et al.* have presented similar data (4).

Precision of the Method Applied to Urine

The precision has been estimated from duplicate determinations of CA in urine. The standard error of a mean of two determinations (i.e. duplicates) is 4.4. ng./minute, based upon analysis of 18 samples. A difference between two such means as great as, or greater than, 13.1 ng./minute would be statistically significant at the 5% level of probability ($t = 2.110$).

Catecholamine Content of Rat Organs

Because of the well known role of the sympathoadrenal system in response to emergencies, of which trauma resulting in death may be one, it was considered important to select a method for sacrificing animals used in physiological experiments which would entail the least influence on the concentration of CA in the adrenal. Such an experiment was performed, with the results given in Table III. Four techniques of killing the rats were used: (*A*) decapitation; (*B*) fracture of the vertebral column in the cervical region; (*C*) administration of ether; and (*D*) intracardial injection of air. Each group contained four rats, and the CA content of the adrenals was determined in duplicate, providing a total of 32 analyses. The analysis of variance (30) in Table III indicates the marked differences in CA content in the four groups (Part A, line 2). Moreover, using the variance between

TABLE III

CONCENTRATION OF CATECHOLAMINES IN RAT ADRENAL GLANDS

A. Analysis of variance

Sources of variation	Degrees of freedom	Mean square	*F*	Significance
All	31	—	—	—
Methods of sacrifice	3	24.54	22.70	<1%
Within methods				
A	3	19.48	18.02	<1%
B	3	3.46	3.20	>5%
C	3	38.13	35.28	<1%
D	3	19.54	18.08	<1%
Remainder (between duplicates)	16	1.08	(1.00)	—

B. CA concentration

Group and means of killing	CA content
A. Decapitation	16.49 μg./pair adrenals
B. Cervical fracture	15.36
C. Ether	12.94
D. Intracardial air	13.06

TABLE IV

CONCENTRATION OF CATECHOLAMINES IN RAT ORGANS

Organ	Concentration, μg./g. of tissue
Liver	0.14
Spleen	0.83
Heart	0.66

duplicate determinations as the measure of error, it is seen that group *B* exhibited uniformity in the CA content of their adrenals, whereas the other methods led to significant variation between animals (Part A, "Within Methods").

Data on the concentration of CA in tissues of male albino Sprague-Dawley rats are given in Table IV. The purification of catecholamines from these organs was performed by the column adsorption method of Weil-Malherbe and Bone (38) before the present extraction procedure was developed; the remainder of the determination was done as described in this paper.

Catecholamine Content of Urine of a Patient with Pheochromocytoma

Through the courtesy of Drs. E. Goldstein and C. Schneiderman of the Jewish General Hospital, Montreal, it was possible to examine several 24-hour urines from a female patient, age 46, proved at operation to have a pheochromocytoma (weighing 500 g.) on the left side. One week before

removal of the tumor the output was 917 μg./24 hours; 2 days before removal it was 880 μg. On the 3rd, 5th, and 6th postoperative days, the excretion rate had fallen to 90, 120, and 59 μg., respectively. The higher values on the first two postoperative days may partially reflect the continuing excretion of *l*-noradrenaline infused in large quantities during the operation.

Acknowledgments

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ISOLATION OF LABELLED DEOXYRIBONUCLEIC ACID PHOSPHORUS BY A MODIFIED SCHMIDT AND THANNHAUSER PROCEDURE[1]

R. Daoust and Catherine E. Stevens Hooper

Abstract

The DNA phosphorus fraction of tissues was isolated by the method of Schmidt and Thannhauser modified so that the DNA phosphorus fraction attains a state of purity satisfactory for studies with radiophosphorus. This procedure was submitted to a series of test experiments. The results suggested that the DNA isolated by the procedure is practically free from contamination by other phosphorus-containing fractions, namely acid-soluble phosphorus, phospholipids, ribonucleic acid, and phosphoproteins. The modified method seems reliable for estimating the specific activity of DNA phosphorus following the administration of radiophosphorus to animals.

Introduction

The method of Schmidt and Thannhauser for measuring the nucleic acid content of tissues (9) does not seek the isolation of DNA in a state of high purity and is not applicable, as such, to studies with radioelements. However, in view of the practical advantages of this procedure over more elaborate methods of DNA preparation, an attempt was made to improve it so as to obtain a DNA P fraction of sufficient purity for radioactive analysis. Several experiments were conducted to determine whether the isolated DNA P was suitable for studies with P^{32}.

Methods

1. Separation of Phosphorus Compounds

The procedure of Schmidt and Thannhauser for fractionating the phosphorus compounds of tissues (9), as outlined in Fig. 1, was modified in several ways, one of which was to separate the extracts from the precipitate by centrifugation rather than filtration and then to wash the precipitate extensively at each step by repeated suspension in the extracting solution, stirring the suspension, and centrifuging it. This procedure is similar to the ones used by Davidson *et al.* (3) and by McCarter and Steljes (7).

Final Technique

(*a*) *Extraction of Acid-soluble Phosphorus*

Three grams of tissue pulp are homogenized with 40 ml. of ice-cold 10% trichloroacetic acid (TCA) in a Potter–Elvehjem type homogenizer. The homogenate is transferred to a 50 ml. centrifuge tube and centrifuged at 1500 r.p.m. until the supernatant fluid is clear of particles (about 5–10

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minutes). The supernatant fluid is drained off. This fraction, the acid-soluble fraction, contains inorganic phosphorus and organic phosphorus compounds which can be separated by the method of Delory (4). The residue is resuspended in 40 ml. of ice-cold 10% TCA, washed carefully by stirring with a glass rod, and centrifuged again. This step is repeated twice more and is followed by two washings with 25 ml. of distilled water. Care is taken not to let the mixtures stand for prolonged periods between the successive washings. The TCA solutions and the water used for washing are discarded.

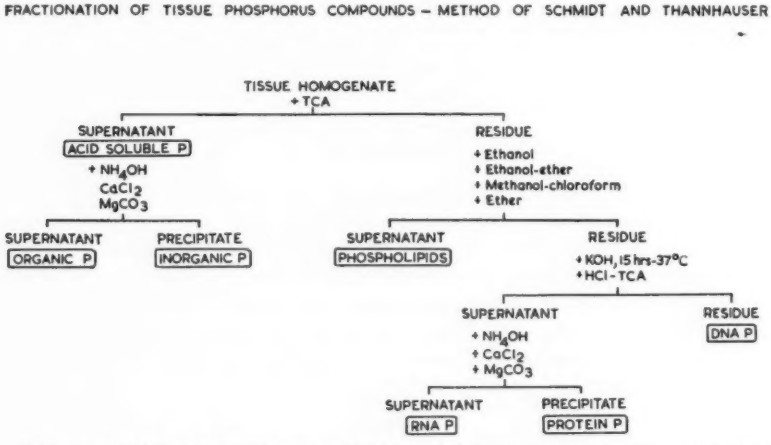

FIG. 1. Outline of the method of Schmidt and Thannhauser for the separation of the phosphorus compounds of tissues.

(*b*) *Extraction of Phospholipids*

Following the last washing with water, the residue is resuspended in 40 ml. of absolute alcohol and the mixture is allowed to stand overnight at 0–5° C. After centrifugation and separation from the supernatant fluid, the residue is extracted with 40 ml. of 1:3 ether–ethanol. The residue is then transferred to an Erlenmeyer flask with 90 ml. of 1:3 ether–ethanol and refluxed for 30 minutes. After cooling, the suspension is transferred to centrifuge tubes and the residue is separated from the supernatant by centrifugation. This step is repeated with 90 ml. of 1:1 methanol–chloroform. The residue is then washed three times by suspension in 25 ml. of ether, stirring, and centrifuging. The washed residue is dried by gentle warming over a hot plate.

(*c*) *Extraction of RNA Phosphorus and Protein-bound Phosphorus*

Thirty milliliters of 1.5 *N* KOH are added to the dry powder and the mixture is incubated at 37° C. for 48 hours. The alkaline hydrolyzate is then filtered to remove the small particles which remain undissolved. The alkaline hydrolyzate is collected in a centrifuge tube and 8 ml. of 20% TCA and 10 ml. of 6 *N* HCl are added. After being allowed to stand

overnight at 0–5° C., the supernatant fluid and the precipitate are separated by centrifugation. The supernatant fluid, which contains the RNA phosphorus in the organic form (acid-soluble nucleotides) and the phosphorus from phosphoproteins in the inorganic form, was drained off. The precipitate containing the *DNA phosphorus* is washed three times by suspension in 30 ml. portions of 1:5 6 *N* HCl – 5% TCA, stirring, and centrifuging.

2. Radioactivity Measurements

For measuring the specific activity of the isolated phosphorus fractions, the extracts are filtered into flasks and reduced by evaporation on a hot plate to a volume of about 2 ml. These solutions, as well as the final residue containing the DNA, are digested with concentrated sulphuric acid and then diluted to known volumes with distilled water. The P^{31} content of these solutions is determined according to the method of Fiske and Subbarow (5). For P^{32} estimations, triplicate plates are prepared by placing 0.5 ml. samples of the same solutions on 20 mm. watch glasses with four drops of 1.5 *N* KOH and two drops of concentrated perchloric acid. These are dried on a hot plate until a fine crystalline film forms and then the radioactivity is measured with a Geiger-Müller tube. The specific activity of the phosphorus fractions is computed as the number of counts per 100 seconds per microgram of phosphorus.

Evaluation of the Method

In order to evaluate the method for isolating labelled DNA P, a series of experiments was carried out in which the separation of each phosphorus fraction (Fig. 1) from the DNA P fraction was considered separately.

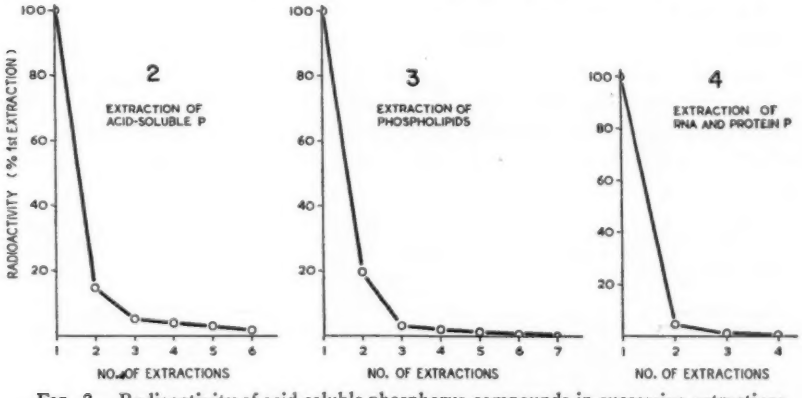

Fig. 2. Radioactivity of acid-soluble phosphorus compounds in successive extractions of tissue homogenate with 10% TCA (extractions 1–4) and distilled water (extractions 5 and 6).

Fig. 3. Radioactivity of phospholipids in successive extractions of the residue with absolute alcohol (1), 1:3 ether–ethanol (2), hot 1:3 ether–ethanol (3), hot methanol–chloroform (4), and three washings with ether (5–7).

Fig. 4. Radioactivity of RNA and phosphoprotein phosphorus in successive extractions of the hydrolyzed residue with 1:5 6 *N* HCl – 5% TCA.

(*a*) *Extraction of Acid-soluble Phosphorus*

The acid-soluble phosphorus is the tissue fraction which shows the highest specific activity at early time intervals (0–24 hours) after the administration of P^{32}. Accordingly, the acid-soluble fraction is considered the most active possible contaminant of the DNA P fraction and its removal must be practically complete. Fig. 2 shows the radioactivity of the successive TCA extracts of liver homogenates prepared from animals injected with P^{32}. It is evident that after the initial drop in the counts of the first three extracts, the radioactivity of the following extracts decreased very slowly. The source of radioactivity in the TCA extracts after the first three is probably the formation of degradation products from acid-insoluble compounds. In fact, when the tissues were allowed to stand in 10% TCA at 0–5° C. overnight after the third extraction, the radioactivity of the following extract was considerably higher than that of the previous ones. This extract also had a higher content of ultraviolet absorbing material, suggesting that part at least of the radioactivity comes from the breakdown of nucleic acids. Another argument supporting this view is that rigorous acid-treatment (following the third extraction) did not appreciably decrease the specific activity of the DNA P fraction, in agreement with the results of other authors (3, 7), whereas it resulted in a considerable loss of DNA P. The acid extractions were thus limited to four, to minimize the degradation of acid-insoluble compounds, and were followed by two washings with distilled water.

To determine whether the DNA P fraction extracted by the above procedure was actually free from acid-soluble contaminants, the following experiment was conducted.

Experiment

A homogenate of rat liver from *uninjected* animals was prepared in Ringer–Locke solution, separated into three parts, and 30 μc. of P^{32} was added to each. The three samples were allowed to stand at 0–5° C. for 5 minutes, 2 hours, and 24 hours respectively, after which the tissue homogenates were fractionated and the specific activities of the acid-soluble and DNA P fractions estimated.

TABLE I

SPECIFIC ACTIVITY OF DNA P FRACTION ISOLATED FROM RAT LIVER HOMOGENATES AT VARIOUS TIME INTERVALS AFTER ADDITION OF P^{32}

Homogenate No.	Time interval*	Specific activity†		Relative specific activity of DNA P§
		Acid-sol. P	DNA P	
1	5 min.	1408	1.17	0.08
2	2 hr.	1332	0.90	0.07
3	24 hr.	1341	0.61	0.05

*Interval between the addition of radiophosphorus to the homogenate and the extraction of the phosphorus fractions.

†Counts per 100 seconds per microgram of phosphorus.

§Percentage ratio of the specific activity of DNA P over the specific activity of acid-soluble phosphorus.

Results.—The results (Table I) showed that the specific activity of the DNA P, expressed as a percentage of that of the acid-soluble phosphorus, was of the same order for the three samples with an average value of 0.07%. A similar ratio of 0.10% was calculated from the results of McCarter and Steljes (7) using their data on rat liver homogenates left in contact with P^{32} for 0, 24, and 48 hours, while a ratio of 0.05% was obtained from recent, unpublished results obtained in Davidson's laboratory with rabbit liver homogenates (private communication). Thus the results obtained in these three laboratories agree that DNA P is contaminated by acid-soluble phosphorus to give a specific activity of DNA P which is 0.05–0.10% of that of the acid soluble fraction. The degree of contamination is not related to the time of exposure and presumably occurs immediately upon homogenization with acid. This degree of contamination would be important in experiments of short duration, but would be negligible, for example, when compared with the amount of P^{32} incorporated into liver DNA during a 24 hour period. The relative specific activity of DNA P at 24 hours after injection is about 3.3% on the average (extreme values: 2.5 and 4.5), i.e. 50 times that which could be attributed to acid-soluble contaminants.

(*b*) *Extraction of Phospholipids*

Following the extraction of acid-soluble phosphorus compounds, the phospholipids were removed from the residue by submitting the latter to seven successive extractions with fat solvents. The radioactivity of the successive extracts (Fig. 3) shows that the removal of phospholipids was practically complete after the fifth extraction. Generally, no appreciable radioactivity could be detected in the sixth and seventh extracts.

Since hot acetone removes some tissue lipids which are not extracted by hot ether (6), the effect of this solvent and also of butanol was examined.

Experiment

Adult albino rats were injected with 300 μc. of P^{32} and sacrificed 24 hours later. The phosphorus fractions were isolated from 9 g. of liver pulp by the method described above. The collected DNA P fraction was separated into three parts. While the first one served as control, the second was further extracted with 90 ml. of hot 1:1 acetone–ethanol for 30 minutes (and washed twice with ether) and the third one was extracted three times with 40 ml. portions of butanol at room temperature. The specific activity of each sample was then determined.

Results.—The control DNA P fraction showed a specific activity of 2.8% of that of the acid-soluble phosphorus, while those submitted to acetone–ethanol and butanol treatments showed relative specific activities of 3.5% and 2.5% respectively. Since no decrease in the specific activity of DNA was observed, these treatments did not improve the purity of the DNA P fraction. The lipids not extracted by ether (6) might not be phospholipids or might be removed by one of the other fat solvents used in the present procedure for lipid extraction.

(c) Extraction of RNA P and Phosphoprotein Phosphorus

Following the extraction of the acid-soluble phosphorus and the phospholipids, the RNA and the protein-bound phosphorus were separated from the DNA P by means of alkaline hydrolysis. This treatment degrades the RNA to acid-soluble nucleotides and liberates the phosphorus of the phosphoproteins as inorganic phosphate. After precipitation of the intact DNA with acid, the supernatant, containing the degradation products of RNA and phosphoproteins, was separated by centrifugation and the precipitate was washed with acid. Fig. 4 shows the radioactivity of the successive acid extracts. Most breakdown products seem to be removed by the first two extractions.

In order to verify whether the DNA P fraction was actually free from acid-soluble degradation products, samples of the DNA P fraction were further purified.

Experiment 1

Adult albino rats were injected with 300 μc. of P^{32} and sacrificed 24 hours later. The DNA P isolated from 12 g. of liver pulp was divided into three parts. The first one was used as control. The other two were redissolved in 1.5 *N* KOH and precipitated with 1:3 HCl–methanol two and five times respectively. The specific activity of each sample was then measured.

TABLE II

SPECIFIC ACTIVITY OF THE DNA P FRACTION AFTER REPEATED PRECIPITATION WITH HCl–MeOH

Sample No.	Number of additional precipitations	Relative specific activity of DNA P*	
		1st experiment	2nd experiment
1	None	3.1	2.9
2	2	2.8	2.8
3	5	—	4.5

*Percentage ratio of the specific activity of DNA P over that of acid-soluble phosphorus.

Results.—The results showed (Table II) that repeated precipitation did not lower the specific activity of the DNA P fraction. It thus seems that the DNA P fraction isolated by the present procedure is practically free of acid-soluble products liberated from RNA and phosphoproteins by alkaline hydrolysis.

Another possibility was that RNA or phosphoproteins were not completely hydrolyzed by the alkali and that a small amount, remaining in the acid-insoluble form, would contaminate the DNA P fraction.

Experiment 2

Adult albino rats were injected with 500 μc. of P^{32} and sacrificed 24 hours later. The liver DNA P fraction was isolated from one animal using 1 *N* NaOH as hydrolytic agent while 1 *N* KOH was used for isolating the DNA

P from a second animal. Each DNA P fraction was divided into two parts, the second being redigested at 40° C. for 18 hours with either NaOH or KOH.

Results.—The DNA P isolated after digestion with NaOH showed a specific activity of 5.0* and, after KOH digestion, a value of 6.7. The samples digested twice showed corresponding values of 7.4 and 7.8. No appreciable difference was thus observed between the specific activities of the samples obtained by either NaOH or KOH digestion and since prolonged incubation periods did not reduce the specific activities, it seems that the treatment did not improve the purity of the DNA P fraction.

The possibility that undigested RNA contaminates the DNA P fraction was especially considered in the following experiment.

Experiment 3

Adult albino rats were injected with 300 μc. of P^{32} and sacrificed 24 hours later. The acid-soluble phosphorus and the phospholipids were extracted from 12 g. of liver pulp as described above. The collected residues, dissolved in 1.5 *N* KOH, were divided into four parts. While the first one served as control, 1.1 g. of commercial (nonradioactive) RNA was added to the second part and 2.2 g. to each of the other two samples. It was calculated that the ratio of labelled to unlabelled RNA was approximately 1:50 in sample 2 and 1:100 in samples 3 and 4. After digestion, the DNA P fractions were isolated by the usual technique and their specific activities were determined.

Results.—The control DNA P fraction showed a specific activity of 4.1% relative to that of the acid-soluble phosphorus, the fraction isolated from sample 2 (RNA dilution 1:50) showed a relative specific activity of 3.8, while those isolated from samples 3 and 4 (RNA dilution 1:100) gave corresponding values of 3.9 and 3.0 respectively. Since the specific activity of tissue RNA was lowered to 1/100th its original value by dilution with nonradioactive RNA, a decrease in the specific activity of DNA should have been observed if the latter had been contaminated to any significant extent by either undigested RNA material or acid-soluble breakdown products of this compound. The fact that no appreciable difference was observed between the control and the RNA-diluted samples thus suggests that the DNA P isolated by the present procedure is practically free from both undigested RNA and degradation products of RNA.

The possibility that DNA P is contaminated by undigested phosphoproteins was examined in the following experiment.

Experiment 4

Adult albino rats were injected with 1 mc. of P^{32} and sacrificed 24 hours later. The DNA P fraction isolated from 9 g. of liver pulp was divided into three parts. The first one served as control. The second part was heated with 5% TCA at 90° C. for 30 minutes (8), after which the supernatant

*It may be pointed out that while the results expressed as "specific activity" (counts per 100 seconds per microgram of phosphorus) are comparable within each experiment, only those expressed as "*relative* specific activity" (percentage ratio of the specific activity of DNA Pover that of acid-soluble phosphorus) are comparable from one experiment to another.

containing the extracted DNA was separated by centrifugation. The third sample was treated with 1 *M* NaCl for 4 hours at room temperature (10) and the supernatant fluid, containing the extracted DNA, was separated by centrifugation. The specific activities of the control and extracted DNA P fractions were then determined.

Results.—While the control DNA P showed a specific activity of 7.8 counts/100 sec./μg. P, the fractions extracted with hot TCA and 1 *M* NaCl showed activities of 9.8 and 6.5 respectively. Since no appreciable decrease in the specific activity of the DNA P was obtained by these treatments, it was considered unlikely that undigested phosphoproteins would remain in the DNA P fraction isolated by the present procedure.

These studies suggest that the DNA P isolated by the modified Schmidt and Thannhauser procedure is practically free from contamination by other phosphorus fractions, namely, the acid-soluble phosphorus, phospholipids, ribonucleic acid, and phosphoproteins. Accordingly, the method seems reliable for estimating the specific activity of DNA P following administration of radiophosphorus to animals (1, 2, 11).

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EFFECT OF HISTAMINE ON GASTRIC PEPSINOGEN, PEPSIN SECRETION, AND BLOOD PEPSINOGEN IN GUINEA PIGS[1]

K. KOWALEWSKI

Abstract

The endocrine and exocrine activity of guinea pig stomach was measured by the determination of pepsinogen in gastric tissue and in plasma. Gastric juice pepsin was also studied.

A significant increase of both pepsinogen and pepsin was found in animals treated with a dose of histamine (75 mg. per kg. of body weight). These results give further evidence that the zymogenic cells of gastric mucosa may be stimulated by histamine. The determination of pepsinogen in gastric tissue seems to permit a direct approach to the enzymatic function of zymogenic cells.

Introduction

The effect of histamine on the gastric endocrine function in guinea pigs (6, 7) and dogs (8, 9, 10) was studied previously in our laboratory. A significant increase of plasma pepsinogen was found in guinea pigs injected with a dose of histamine sufficient to induce gastric ulcers (6). In dogs the elevation of posthistaminic plasma pepsinogen was accompanied by increased production of gastric pepsin (9). It was also observed (9) that the pepsinogen concentration was higher in the gastric vein than in the gastric artery in dogs, and that this difference was accentuated following histamine administration. These findings were attributed to endocrine secretion of pepsinogen by the zymogenic cells of gastric mucosa and it was apparent that histamine influenced this secretion. This indirect evidence of action of histamine on the pepsin secreting cells seemed to warrant further investigation.

It is apparent from a recent report (3) that the gastric tissue pepsinogen may be measured directly in homogenized stomachs of rats. This method of study of gastric enzyme seems to permit a direct approach to the function of zymogenic cells.

In the present study an attempt was made to determine the pepsinogen content of the gastric tissue of guinea pigs and to correlate it with the levels of this enzyme in blood and gastric juice, before and after treatment with histamine.

Experimental

Male guinea pigs fed on commercial pellets and vegetables, and having a weight range of 400 to 520 g., were used. Forty-eight hours before the beginning of the study the animals were put in separate cages and allowed water only. Guinea pigs sacrificed without treatment served as a control group. The other animals received subcutaneous injections of Phenergan (Poulenc) in a dosage of 30 mg. per kilogram of body weight, followed in 30 minutes by intramuscular injection of histamine dihydrochloride (Roche) in a dosage of

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Contribution from McEachern Cancer Research Laboratory and the Department of Surgery, University of Alberta, Edmonton, Alberta.

TABLE I

EFFECT OF HISTAMINE ON GASTRIC TISSUE AND PLASMA PEPSINOGEN AND ON VOLUME, ACIDITY, AND PEPSIN OF THE GASTRIC CONTENT IN GUINEA PIGS. THE RESULTS ARE EXPRESSED IN TERMS OF THE AMOUNT OF TYROSINE RELEASED BY THE PROTEOLYTIC ACTION OF A MILLILITER OF PLASMA, OF THE TOTAL GASTRIC TISSUE, AND OF THE TOTAL GASTRIC JUICE. FREE HYDROCHLORIC ACID OF GASTRIC JUICE IS EXPRESSED IN MEQ. PER TOTAL GASTRIC CONTENT

					Gastric juice		
Animals sacrificed	No. of animals	Values	Total gastric tissue pepsinogen, mg.	Plasma pepsinogen, μg./ml.	Vol., ml.	Total free HCl, meq.	Total pepsin, mg.
Fasting no treatment	12	Mean	554.0	58.0	5.0	0.4	14.0
		S.D.	±75.0	±8.7	±1.4	±0.16	±4.0
		Range	421.4–629.0	46.0–72.0	3.8–8.0	0.2–0.7	8.0–19.5
One hour after histamine	12	Mean	820.0	122.0	9.8	1.1	43.0
		S.D.	±61.8	±27.2	±1.6	±0.27	±7.9
		Range	733.0–948.2	96.0–184.0	7.0–11.5	0.5–1.4	29.9–52.8
Two hours after histamine	12	Mean	890.0	215.0	12.8	1.4	115.0
		S.D.	±52.6	±32.1	±1.1	±0.32	±30.9
		Range	829.2–963.2	163.0–290.0	11.7–15.5	0.9–2.0	73.4–173.9
Three hours after histamine	12	Mean	1106.0	309.0	14.7	1.4	97.0
		S.D.	±132.3	±25.2	±1.1	±0.42	±20.1
		Range	854.2–1315.0	265.0–349.0	13.0–16.5	1.0–2.2	75.4–140.2
Four hours after histamine	12	Mean	1002.0	340.0	14.9	1.6	89.0
		S.D.	124.8	54.3	2.0	0.32	11.8
		Range	804.6–1230.0	290.0–452.0	12.5–20.0	1.0–2.1	76.4–110.4

75 mg. per kilogram of body weight. As shown previously, the antihistamine protects the animals against the systemic effect of this high dose of histamine (2, 4, 11).

The injected animals were sacrificed in groups, 1, 2, 3, and 4 hours after the administration of histamine. The blood was collected from the inferior vena cava, under ether anesthesia, in oxalated vacuum tubes. The stomachs were removed and their contents collected for the determination of free acid and pepsin. Each stomach was opened along the greater curvature, carefully washed under a stream of running distilled water, and homogenized individually in 100 ml. of 0.1 *N* hydrochloric acid in a Waring blendor for 10 minutes. The blendor was then washed with an extra 50 ml. of the acid, the total suspension filtered, and aliquots were taken for the pepsinogen study.

Plasma pepsinogen was determined according to the procedure of Mirsky *et al.* (12), but plasma was digested with the substrate for 20 hours and not for 24 hours as in the Mirsky technique.

Pepsin was determined on gastric juice and pepsinogen on the filtered homogenized gastric tissue, following the procedure of Anson as described by Aitken *et al.* (1) except that a 2.5% solution of hemoglobin was used as substrate (9).

The results are expressed in terms of the amount of tyrosine released by the proteolytic action of a milliliter of plasma, of the total gastric tissue, and of the total gastric juice found at the moment of sacrificing the guinea pig. Free hydrochloric acid of gastric juice is expressed in milliequivalents per total gastric content.

Results and Conclusion

Table I and Fig. 1 summarize the results.

It may be seen that the gastric tissue is rich in the enzyme considered to be pepsinogen of zymogenic cells. The amount of pepsinogen in the gastric tissue is several times higher than the amount of pepsin in fasting gastric content and several thousand times higher than that of pepsin in 1 ml. of plasma. This finding may indicate that the substance found in gastric tissue is the source of the peptic enzyme measured in gastric juice and in blood. In this way the hypothesis of both exocrine and endocrine function of zymogenic cells finds further verification.

Histamine produces a significant rise of pepsinogen (gastric tissue and blood) and pepsin (gastric juice) and this rise was observed in all guinea pigs studied at various times after the beginning of treatment. The posthistaminic changes found in volume and acidity of gastric juice are similar to those previously reported (9). These results give further evidence that both exocrine and endocrine function of gastric mucosa may be stimulated by histamine.

The present and previous experiments (6, 9, 10) permit only this conclusion as far as exogenous histamine is concerned. It is known, however, that gastric mucosa is rich in histamine which may be extracted and used as a gastric stimulant (13). Our work shows that gastric mucosa is rich in pepsinogen and that histamine stimulates the production of this enzyme by

zymogenic cells. An interesting speculation is the possibility that the endogenous histamine is a factor which regulates the peptic activity of gastric mucosa, especially after some stress stimuli.

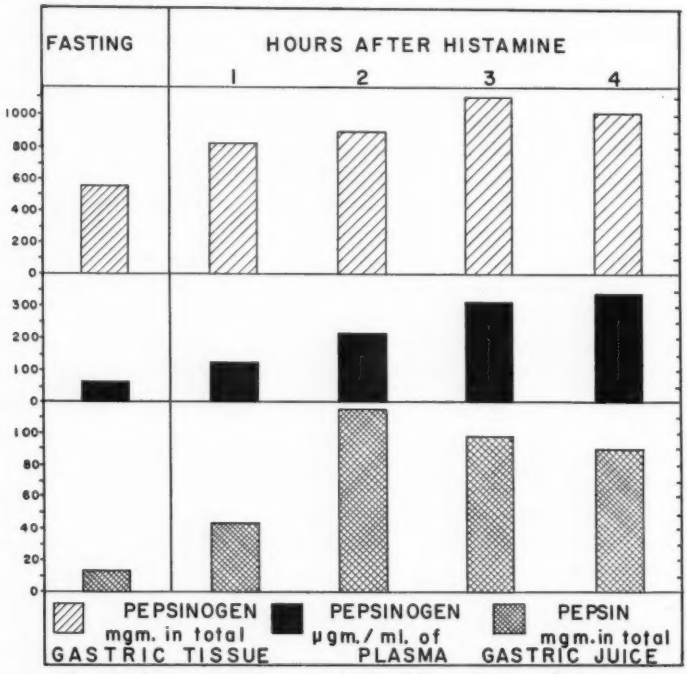

FIG. 1. Gastric tissue pepsinogen correlated with pepsinogen of plasma and pepsin of gastric juice. Average values for 12 guinea pigs in each of five groups.

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THE MECHANISM OF DEGRADATION OF CELLULOSE BY MYROTHECIUM CELLULASE[1]

D. R. WHITAKER

Abstract

A swollen cellulose was hydrolyzed by *Myrothecium* cellulase and the loss in weight, number-average degree of polymerization, and weight-average degree of polymerization were determined at various intervals. The loss in weight relative to the change in D.P. excludes endwise cleavage as the mechanism of hydrolysis. An approximate test for random cleavage is derived from the Beall–Jorgensen treatment of random cleavage processes and, within the limits of this test, the change in weight-average D.P. relative to the change in number-average D.P. is shown to be consistent with random cleavage.

Introduction

Previous reports from this laboratory have discussed the method by which *Myrothecium* cellulase degrades the soluble β-1,4′-oligoglucosides with degrees of polymerization (D.P.) between two and six (32, 33, 34) and a cellodextrin with a number-average D.P. of 24 (35). As an indication of relative orders of magnitude, the hydrodynamic properties of the enzyme correspond to those of a molecule with the same length as a cellulose chain containing about 40 anhydroglucose units (31). The above two groups of substrates thus represent chains whose lengths are respectively much less than and comparable to the length of the enzyme molecule. This report discusses the method of degradation of chains in the remaining category, i.e. cellulose chains with lengths greatly exceeding the length of the enzyme molecule.

Cellulases are generally assumed to degrade chains of this size either by random cleavage or by splitting cellobiose units from the ends of the chains. Several types of evidence may be cited as favoring the first mechanism for *Myrothecium* cellulase: the insensitivity of the rate of hydrolysis to the initial average D.P. of partially hydrolyzed celluloses (9), the reduction in viscosity of soluble derivatives of cellulose before the reducing sugar concentration becomes appreciable (18), the high degree of substitution necessary to protect certain derivatives of cellulose from enzymatic attack (25), and evidence of the formation of higher oligoglucosides during the hydrolysis of cellulose (11, 16). However, none of this evidence is conclusive and the relevance of some of it has been questioned (23).

The evidence reported in this paper consists primarily of data on the loss in weight, the change in number-average D.P., and the change in weight-average D.P. of a swollen cellulose during enzymatic hydrolysis. It enables one of the above mentioned mechanisms to be excluded and the other to be tested approximately by a method described in the following section.

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Theoretical

The most general criteria for testing whether a degradation of cellulose is consistent with random cleavage have been deduced by Beall and Jorgensen (2). They also outlined a method for obtaining the data necessary to test their criteria rigorously. The method is laborious and so far has been applied only to the degradation of cellulose by various inorganic catalysts (13). Some complications presented by an enzymatic hydrolysis and a simple, though less rigorous, method of applying their criteria will now be discussed.

Beall and Jorgensen characterize the length of a chain by the number of glucosidic linkages, λ, in the chain. The initial distribution of chain lengths is characterized by the absolute moments, μ_1, μ_2, etc., of the frequency distribution. They then show, *inter alia*, that if this initial set is randomly hydrolyzed to give a degraded set of chains with corresponding moments designated m_1, m_2, etc., the two sets of moments are related as follows:

$$[1] \qquad m_1 = (1 + p\mu_1)^{-1} q\mu_1 ,$$

$$[2] \qquad m_{2\,p\to 0} = (1 + p\mu_1)^{-1} \left(q\mu_1 + q^2\mu_2 - \frac{2}{3!} pq^2\mu_3 + \frac{2}{4!} p^2q^2\mu_4 - \frac{2}{5!} p^3q^2\mu_5 + \ldots . \right) ,$$

where p is the probability of a given linkage being broken during the period of hydrolysis and $q = 1 - p$ is the probability of the linkage surviving. To apply these equations, p is estimated by equation [1]. The test for random cleavage then resolves into determining whether equation [2] adequately predicts m_2. Each interval in a time sequence can be treated similarly, the degraded product of one interval becoming the initial product of the next.

These equations apply rigorously to an ideal random hydrolysis uncomplicated by accessibility factors or end effects. An enzymatic hydrolysis is subject to both these complications and, because of the greater size and specificity of enzymes, to a greater degree than a hydrolysis by most inorganic catalysts. The accessibility of cellulose to a large molecule is limited not only by the crystallinity of the cellulose but by other elements of fiber structure, e.g. by the capillary network between the microfibrils. Thus usually only a small fraction of the cellulose in a natural fiber is readily accessible to cellulases, this fraction is degraded early in the hydrolysis, and, as clearly shown by Walseth's data (29), the course of the hydrolysis then becomes grossly complicated by accessibility factors. Beall and Jorgensen point out that complications from accessibility factors can be minimized by choosing the reaction intervals so that p is very small. However, the experimental error of the methods used to estimate the moments puts a limit on this choice and, within this limit, their equations are likely to apply to an enzymatic hydrolysis only during the initial stages of the hydrolysis of a cellulose which has been ground and swollen or otherwise treated to increase its accessibility to large molecules. End effects will be present whenever the hydrolytic agent is

sensitive to differences between the properties of terminal and interior linkages but they are likely to cause serious complications only with chains that are so short that terminal linkages represent an appreciable fraction of the total linkages. The soluble oligoglucosides are in this category. Their rates of hydrolysis by *Myrothecium* cellulase increase so sharply with each increase in D.P. (34) that soluble products must be excluded from consideration if equations [1] and [2] are to be applied to a hydrolysis of cellulose. The equations make no allowance for loss of monomer units. Their application must be restricted therefore to intervals in which the loss in weight of the cellulose is small enough to be neglected.

The higher moments in equation [2] present another problem. The moments are defined by the general term:

$$[3] \qquad \mu_r = \sum_{\lambda=0}^{\lambda=\infty} \lambda^r \phi_\lambda \Big/ \sum_{\lambda=0}^{\lambda=\infty} \phi_\lambda$$

where ϕ_λ is the relative frequency of chains of length λ. The first three moments can be estimated in principle from the equations:

$$[4] \qquad \mu_1 = \overline{\text{D.P.}}_{\text{N}} ,$$

$$[5] \qquad \mu_2 = \mu_1 . \overline{\text{D.P.}}_{\text{W}} ,$$

$$[6] \qquad \mu_3 = \mu_2 . \overline{\text{D.P.}}_{\text{Z}} ,$$

where $\overline{\text{D.P.}}_{\text{N}}$ is the number-average D.P. as given by an analysis for reducing end-groups or, if the cellulose is converted without degradation to cellulose nitrate, by osmotic pressure measurements; $\overline{\text{D.P.}}_{\text{W}}$ is the weight-average D.P. as given by suitably calibrated viscosity measurements on the nitrate; and $\overline{\text{D.P.}}_{\text{Z}}$ is the Z-average D.P. as given by appropriate analysis of data obtainable with the equilibrium ultracentrifuge or by light scattering photometry. However, equation [6] is of little value as both methods of estimating $\overline{\text{D.P.}}_{\text{Z}}$ directly are limited in practice to cellulose nitrates with very low values of $\overline{\text{D.P.}}_{\text{Z}}$ (14, 4). Beall and Jorgensen estimated the third and higher moments by an indirect method. They fractionated each sample of cellulose into a large number of fractions, determined the first two moments on each fraction, and then computed the required moments from these data with a mechanical integrator. This method is very laborious and the authors express reservations with respect to its accuracy.

A less rigorous method is followed in this paper. Firstly, by using a substrate with not too high a molecular weight ($\overline{\text{D.P.}}_{\text{W}} = 1000$) and by keeping p fairly low (between 2×10^{-4} and 9×10^{-4}), the infinite series in equation [2] will be made to converge quite rapidly. It will then be assumed, subject to examination later, that the higher moments of each initial frequency distribution can be approximated by the higher moments of the Gaussian distribution defined by the first two moments. The third and fifth central moments of a Gaussian distribution are equal to zero and the fourth central moment is a simple function of the first two absolute moments; the corresponding

absolute moments are thus readily calculated from the following equations (15) relating central and absolute moments:

$$[7] \qquad \mu_3^C = \mu_3 - 3\,\mu_2\mu_1 + 2\,\mu_1^3,$$

$$[8] \qquad \mu_4^C = \mu_4 - 4\,\mu_3\mu_1 + 6\,\mu_2\mu_1^2 - 3\,\mu_1^4,$$

etc.

where the superscript C denotes central moments, i.e. moments taken about the arithmetic mean.

Materials

The substrate was a finely dispersed, swollen cotton cellulose. It was prepared from cotton linters (Grade 27, Hercules Powder Co.; $\overline{\text{D.P.}}_{\text{w}}$ *ca.* 2000) which had been ground in a Wiley mill to pass a 1 mm. mesh sieve, extracted with benzene–alcohol (2:1 v/v), and vacuum dried. The ground linters (75 g.) was swollen and partially degraded by treatment for 4 hours with 1 liter of 85% phosphoric acid at 0°. It was then stirred into 4 liters of ice water, filtered off, dispersed in cold water in a Waring blendor, washed on the filter with 20 liters of ice water, and left overnight dispersed in 0.01 *M* sodium acetate. The cellulose was rewashed on the filter for several days with distilled water and then redispersed in the blendor. The dispersion was freed of coarse aggregates by filtration through a No. 24 mesh sieve and diluted with water to contain approximately 2 g. of cellulose per 100 g.

The enzyme was obtained from culture filtrates of the mold *Myrothecium verrucaria* and purified as previously described (30). It was freed of salts by an ion-exchange procedure which will be described in a subsequent paper. All chemicals were of reagent grade. Organic solvents were redistilled through a Stedman column.

Methods

1. Enzymatic Hydrolysis

The dispersion of cellulose was brought to a pH of 5.0 and an ionic strength of 0.02 with acetic acid and sodium acetate. After the addition of solutions containing enzyme and bovine plasma albumin (Armour) at the same pH and ionic strength, 100 g. of dispersion contained 1.92 g. of cellulose, 2.0 mg. of enzyme, and 2.0 mg. of albumin. The dispersion was gently shaken and its temperature maintained at 24.8° C. throughout the reaction period of 24 hours. Microscopic examination of the final hydrolyzate showed no evidence of bacterial contamination.

2. Treatment of Samples

Two samples were removed at various intervals. The first, *ca.* 25 ml. in volume, was used to determine the total number of reducing end groups in the dispersion, i.e. end groups in solution plus end groups in the residual cellulose. It was quickly weighed in a tared flask and then frozen in a bath of acetone and dry ice to stop the reaction. The reagents for the aldose oxidation procedure of Martin *et al.* (20) were added and the iodine consumption measured after reaction for 3 hours at 0° C.

The second sample, *ca.* 200 ml. in volume, was used for all measurements on the residual cellulose. After the sample was weighed, the reaction was stopped by adding 200 ml. of hot ethanol and boiling the mixture 5 minutes. To remove soluble products, the dispersion was centrifuged for 20 minutes at 32,000 *g* and the sediment was redispersed in 200 ml. of 50% aqueous ethanol and recentrifuged. Traces of residual cellulose in the supernatant solutions were removed by filtration through a tared, fine-porosity, sintered glass filter. The sediment was washed on the filter with absolute ethanol, air-dried, and then dried to constant weight over phosphorus pentoxide in a high vacuum drying pistol at 80° C. As a check on the completeness of removal of soluble sugars, 10 mg. of each residual cellulose was extracted with water and the extract developed on paper chromatograms of a type previously described (32). The only sugar detectable was cellobiose, which appeared as a faint trace on the chromatograms representing periods of enzymatic hydrolysis in excess of 1 hour.

Reducing end-groups in the residual celluloses were determined on 1 g. samples by the method of Martin *et al.* (20). To prevent interference by traces of adsorbed ethanol, the samples were first equilibrated over water in a closed vessel for 48 hours.

A second 1 g. sample was converted to cellulose nitrate by nitration for 1 hour at 0° C. in 50 g. of a solution of nitric acid, phosphoric acid, and phosphorus pentoxide (64: 26: 10 w/w) which was prepared as described by Alexander and Mitchell (1). These reaction conditions are now recognized to cause little or no degradation of cellulose (10, 3, 27). The nitrate was washed and stabilized by a procedure differing from that of Timell and Purvess (26) only in that the sample was washed with ice water prior to washing with cold aqueous ethanol. The final product was collected on a tared sintered glass filter, air-dried, and then dried overnight in vacuum over phosphorus pentoxide. The nitrogen contents, determined as described by Timell and Purvess (26), ranged from 13.5 to 14.1%. The weights of the products were within 1% of the theoretical values calculated from the weight of cellulose nitrated and the nitrogen analysis.

3. *Physical Measurements on the Cellulose Nitrates*

(*a*) *Osmotic Pressure Measurements*

Osmotic pressures of solutions of the nitrates in ethyl acetate were measured by the equilibrium method as described by Flory (8). The osmometer was of the Fuoss and Mead type (7), modified to incorporate certain modifications of Sirianni *et al.* (24) for the prevention of leakages. The membrane was prepared by denitrating a collodion membrane by the method of Montonna and Jilk (21). It was conditioned to ethyl acetate by immersion for several hours in ethanol water (1: 1), ethanol, ethanol – ethyl acetate (1: 1), and finally for several days in ethyl acetate. Temperature control during the measurements was provided by an oil bath at 27.7 ± 0.02° C. To prevent

bubble formation at this temperature, the osmometer was filled in a room at 30° C. and, as a further protection against leakage, the drainage tubes and the screws closing them were sealed with a "liquid solder" cement.

The number-average D.P. was calculated from the equation $\overline{D.P.}_N = M_n/m$ where M_n is the molecular weight estimated from the osmotic pressure measurements and m is the weight of the monomer unit given by the nitrogen analysis.

The osmotic pressure measurements were limited to the first few reaction intervals as the membranes employed proved to be too permeable to give steady osmotic pressures with the more highly degraded celluloses.

(*b*) *Viscosity Measurements*

The viscosities of solutions of the nitrates in ethyl acetate were measured at 24.8° C. in capillary viscometers. The viscometers were basically of Cannon–Fenske design (5) but were provided with additional bulbs to give readings at three mean velocity gradients. The gradients for each bulb were estimated by Kroeplin's equation (17):

$\overline{G} = \frac{8\ V}{3\ \pi\ r^3\ t}$, where $\overline{G}$ is the gradient, V is the volume of the bulb, r is the radius of the capillary, and t is the flow time. Viscosities were measured at five concentrations of each nitrate and at each concentration in two viscometers, chosen whenever possible to give a range of gradients bracketing 500 seconds^{-1}.

The data were treated as outlined by Timell (28). The reduced viscosity at a mean gradient of 500 seconds^{-1} was estimated graphically for each concentration by plotting the reduced viscosity, $(\eta - \eta_0)/\eta_0 c$ where η and η_0 are the viscosities of the solution and solvent respectively and c is the concentration of the solute in g./100 ml., as a function of the gradient. The corresponding intrinsic viscosity was then estimated by plotting the logarithm of the reduced viscosity as a function of concentration and extrapolating to zero concentration. Finally the results were corrected to the values for a cellulose nitrate with a nitrogen content of 13.6% by the equation of Lindsley and Frank (19).

These operations give the intrinsic viscosity which Newman, Loeb, and Conrad (22) calibrated in terms of $\overline{D.P.}_{WW}$ (the average given by sedimentation and diffusion measurements). Their equation is

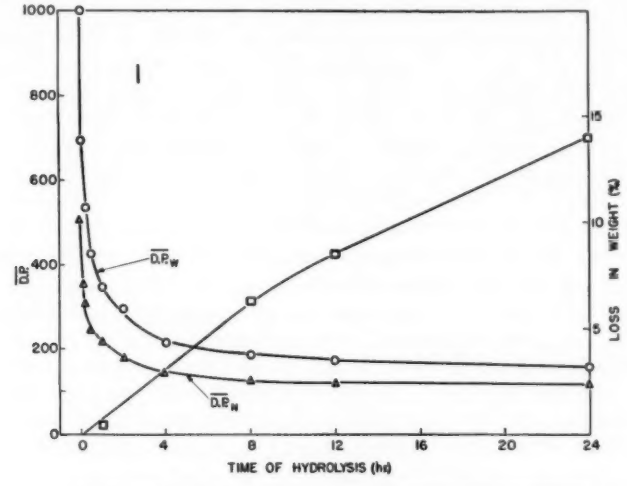
$$\overline{D.P.}_{WW} = 80\ [\eta]_{500\ \text{seconds}^{-1}}$$

when ethyl acetate at 25° C. is the solvent. A corresponding equation for $\overline{D.P.}_W$:

[9] $$\overline{D.P.}_W = 107\ [\eta]_{500\ \text{seconds}^{-1}}$$

was derived from their data by applying Jullander's analysis (14) of the relation between $\overline{D.P.}_N$, $\overline{D.P.}_W$, and $\overline{D.P.}_{WW}$ for cellulose nitrate. The equation assumes that the frequency distribution of their cellulose nitrates satisfied certain conditions but is unlikely to be in serious error. Virtually the same

equation is obtained by a calibration based on the number-average D.P. of the substrate used in the present work and the results of a recent survey by Holtzer, Benoit, and Doty (12) on the calibration of viscosity data for cellulose nitrate.



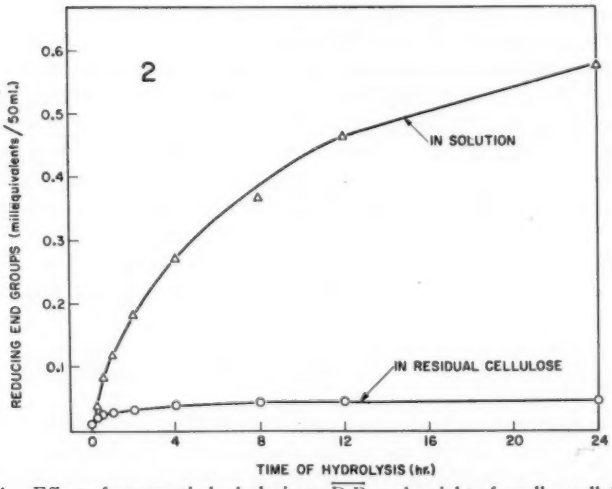


FIG. 1. Effect of enzymatic hydrolysis on $\overline{D.P.}$ and weight of swollen cellulose.
FIG. 2. Reducing end-groups formed during enzymatic hydrolysis of swollen cellulose.

Results and Discussion

The main features of the 24 hour hydrolysis are shown in Figs. 1 and 2. The initial stages are marked by a sharp drop in $\overline{D.P.}_W$ and $\overline{D.P.}_N$ before the loss in weight becomes appreciable. This combination of effects excludes

endwise cleavage as the mechanism of degradation. As hydrolysis continues, both the rate of fall in D.P. and the net rate of hydrolysis decrease. By the final stages, any tendency of further cleavage to lower the $\overline{D.P.}$ is nearly balanced by the passage of short chains into solution. These main trends are similar to those obtained with a swollen cellulose by Walseth (29), who further showed that the moisture regain, a measure of the accessibility of cellulose to water vapor, decreased as degradation continued. Complication by accessibility factors may then be fairly serious in the terminal stages of the hydrolysis but the results show no evidence, such as that obtained by Walseth with unswollen cellulose, of serious complication in the initial stages.

The first and second moments at six periods during the first 2 hours are given in Table I. The experimental second moments were obtained by equation [5], the calculated second moments by equations [1] and [2]. Except at one period, the two values agree within 10%, the limit within which Beall and Jorgensen consider agreement to be satisfactory. The two methods used to estimate the first moment are in good agreement, and, within reasonable limits, any systematic error in the estimate of $\overline{D.P.}_w$ by equation [9] will affect the calculated and experimental moments to approximately the same extent. A remaining systematic influence which might bias the results is the approximation made in applying equation [2] that the frequency distributions are Gaussian. The following discussion of this point was made possible by a statistical analysis of the data by Dr. J. W. Hopkins.

TABLE I

FIRST AND SECOND MOMENTS DURING HYDROLYSIS OF CELLULOSE

Time, min.	First moment $\times 10^{-2}$	Second moment		Deviation, %
		Exptl. $\times 10^{-4}$	Calc. $\times 10^{-4}$	
0	5.08*	50.8*		
5	3.55*	24.7*	27.1	9
	3.63	25.2		
15	3.14*	16.8*	20.0	16
	3.12	16.7	19.6	15
30	2.46	10.5	11.3	7
60	2.20	7.61	7.85	3
120	1.81	5.39	5.56	3

*First moment estimated by osmotic pressure measurement; all other first moments estimated by aldose oxidation.

The initial frequency distributions may deviate from the assumed Gaussian distributions by being skewed, flattened, peaked, etc. The frequency distributions determined by Jorgensen (13) for cotton cellulose range from a strongly negatively skewed distribution for undegraded cotton ($\overline{D.P.}_w = 4000$) to a moderately positively skewed distribution for cotton degraded to a $\overline{D.P.}_w$ of 1000 by treatment with phosphoric acid. Skewness will be reflected in the third moment, flatness or peakedness in the fourth. However, the convergence of the series in equation [2] for the present data is such that only skewness need be considered. An indication that the frequency distributions

were not Gaussian was obtained as follows. For reasons already discussed, soluble chains, i.e. chains with a D.P. up to about ten, were excluded from consideration in applying equations [1] and [2]. This omission, which becomes increasingly important as degradation proceeds, can be allowed for by treating the assumed Gaussian distributions as Gaussian distributions truncated at a D.P. of 10. However, this leads to an impossibility, for if the moments of the parent Gaussian distributions are calculated by Cohen's treatment (6) of truncated distributions, moments are obtained which require the existence of an appreciable number of chains of negative length. This result suggests that the distributions were, in fact, positively skewed.

Skewness is usually measured by the coefficient, γ_1, defined by the equation:

$$\gamma_1 = \mu_3{}^C/(\mu_2{}^C)^{3/2}$$

where $\mu_3{}^C$ is the third central moment, which is related to the absolute moments by equation [7], and $\mu_2{}^C$ is the second central moment, which is related to the absolute moments by the equation:

$$\mu_2{}^C = \mu_2 - \mu_1{}^2 .$$

In Table II, the calculated first and second moments for the first and last interval in Table I are recalculated for frequency distributions with varying degrees of skewness. The table includes degrees of skewness which are physically incompatible with the experimental data, e.g. in order for the frequency distribution at zero time to have a skewness of -5, it would have to contain many chains with large negative lengths, but the extreme range chosen illustrates the degree to which the numerical results at the two intervals differ in their sensitivity to skewness. The infinite series in equation [2] converges increasingly rapidly with each succeeding interval in Table I and by the last interval, the third moment contributes little to the calculation. Within less extreme limits of skewness, the approximation used in Table I does not lead to serious errors. With positively skewed distributions, it will tend to overestimate the calculated second moment and, as shown in Table II, allowance for a moderate positive skewness improves the agreement between the calculated and experimental second moments.

TABLE II

SECOND MOMENTS CALCULATED FOR VARIOUS DEGREES OF SKEWNESS

γ_1 at preceding interval	Calculated second moment			
	At 5 min., $\times 10^{-4}$	Deviation from exptl., %	At 120 min., $\times 10^{-4}$	Deviation from exptl., %
−5	39.4	+60	6.13	+14
−3	34.4	+40	5.89	+ 9
−1	29.5	+20	5.64	+ 5
0	27.1	+10	5.56	+ 2
1	24.6	0	5.39	0
3	19.7	−20	5.15	− 2
5	11.7	−50	4.80	−11

It is concluded from the foregoing evidence that the enzyme degrades cellulose chains representing the third range of chain lengths by essentially random hydrolysis. The main trends shown over three ranges may be summarized as follows. Hydrolysis in first range, that of the soluble oligoglucosides, is marked by a diminishing influence of end effects as chain length increases: hydrolysis of cellobiose is extremely slow; hydrolysis of cellotriose is moderately fast, cleavage of the linkage adjacent to the non-reducing end of its chain is favored; cleavage of cellotetraose is still more rapid, cleavage of its central linkage is favored; hydrolysis of cellopentaose and cellohexaose is extremely fast, deviations from random cleavage are less pronounced (32, 33, 34). Chains in the second (35) and third category of sizes are randomly degraded.

Acknowledgment

The writer wishes to express his thanks to Dr. J. W. Hopkins and Mr. P. P. F. Clay for advice and assistance, to Mr. C. A. McDonell for technical assistance, and to Canada Foils Ltd., Toronto, Canada, for a gift of composition foil used in the construction of the osmometer.

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THROMBIN PROPERTIES[1]

EDWARD RONWIN

Abstract

The enzymatic properties of thrombin have been examined and compared with those of two related enzymes, plasmin and trypsin. The effects of factors such as pH, substrate specificity, ionic strength, cations, anions, and organic reagents on the enzymatic activity of thrombin have been studied. While the three enzymes discussed possess differences, such similarities as were observed are quite striking and permit their classification into one group as tryptic enzymes.

Introduction

In many previously reported investigations of the enzymatic properties of thrombin, the substrate usually studied was fibrinogen. However, fibrinogen has several undesirable qualities which negate its value in quantitative studies. Recently, the simple substrate, N^{α}-*p*-toluenesulphonyl-L-arginine methyl ester (TAME) was introduced (11) and, as it lacks the disadvantages of fibrinogen, it has been employed in this work. A broad study of the properties of thrombin has been carried out and comparisons have been made with related enzymes.

Materials and Methods

Measurement of Enzyme Action and Standard Conditions

The measurement of the hydrolytic activity was made by a continuous titration procedure in the presence of a nitrogen gas flush of the reaction mixture and under the following standard conditions: initial substrate concentration, 0.01 *M*; temperature, 38° C.; pH, 8.0 ± 0.05; buffer, 0.015 *M* Veronal; ionic strength, 0.025; reaction volume, 2 ml. In most cases sufficient substrate and other components as solids or solutions, except enzyme, were mixed to give 3-ml. solutions having the desired concentrations of all substances. Two milliliters of this solution was taken for the enzyme–substrate reaction and the remaining milliliter served as substrate control. The enzyme was stored as a concentrated solution in the refrigerator (comments on its stability under various conditions of storage are given in the section on Results and Discussion) and quantities of 5 to 15 μl., measured by micropipette, were sufficient when added to the 2 ml. of substrate solution to give adequate enzyme concentrations. The time of addition of the enzyme to the substrate was taken as the zero point of the reaction. At this time a small quantity of concentrated base (usually 0.01 ml. of 0.1 *N* NaOH) was added to the reaction mixture with shaking and the time for neutralization of the added base was recorded. The neutralization point was read on the galvanometer of a Beckman model G pH meter. As soon as neutralization occurred this process was repeated. Several points were read for any one reaction run.

[1]Manuscript received March 25, 1957.
Contribution from the Department of Pharmacology, University of Southern California, School of Medicine, Los Angeles 7, California.

Enzyme Preparation

Thrombin, topical (Parke, Davis & Co.), lot no. 029342-B was used throughout. Some of the experiments involving cation inhibition studies were checked with a thrombin preparation from material of lot no. 030229-B and the agreement of results was excellent.

Substrates and Inhibitors

The following substrates were used: N^{α}-*p*-toluenesulphonyl-L-arginine methyl ester hydrochloride (TAME); L-arginine methyl ester dihydrochloride (AMED); N^{α}-3,5-dinitrobenzoyl-L-arginine methyl ester hydrochloride (3,5-DNBAME); N^{α}-*m*-nitrobenzoyl-L-arginine methyl ester hydrochloride (MNBAME); N^{α}-benzoyl-L-arginine methyl ester carbonate (converted to the hydrochloride prior to use) (BAME). TAME was purchased from H & M Chemical Co., Santa Monica, Calif. AMED was an Eastman Kodak product. The other compounds were generous gifts from Dr. A. K. Balls.

The following compounds were employed in inhibition studies: the compounds listed in Table V, all of which were Eastman Kodak products except N-*p*-toluenesulphonylglycine (synthesized by McChesney and Swann's method (3)); N^{α}-*p*-toluenesulphonyl-L-arginine (H & M Chemical Co.); N^{α}-benzoyl-L-arginine and N^{α}-*m*-nitrobenzoyl-L-arginine (from Dr. A. K. Balls); and N-chloroacetyl-L-aspartic acid (synthesized by direct acylation (5)).

Results and Discussion

pH Optimum of Thrombin

This study was undertaken, in part, to establish the pH optimum values under the conditions reported here and, in part, to check on the report of Sherry and Troll (11) that the pH optimum in tris-(hydroxymethyl)-aminomethane (tris) was at 9, in borate at 8, and in phosphate at 7.6.

Fig. 1 gives the pH curves for thrombin in tris, borate–borax, and veronal buffers. (In addition, two comparisons were made in phosphate buffer: at pH 7.60 there was 32.5% hydrolysis of TAME after 5 minutes of reaction and at pH 7.95 there was 45.2% hydrolysis.) In all cases the pH optimum is at 8 and the results differ from those of Sherry and Troll. The tris buffer concentration these authors used was duplicated here (curve B, Fig. 1). The only effect of the higher buffer concentration was to lower the hydrolysis rate (curves A and B, Fig. 1), but the pH optimum and curve shape are virtually identical. The difference is explained by the fact that TAME is spontaneously hydrolyzed at a rate dependent on pH (6). At pH 8 the spontaneous hydrolysis of TAME is negligible whereas at pH 9 it is quite significant. Thus, when the pH curve in tris buffer given by Sherry and Troll (11) (see insert in Fig. 1) is corrected for the spontaneous hydrolysis of TAME at the higher pH levels, the right-hand part of the curve then takes the shape indicated by the dashed–crossed line. Now the curve very closely approximates the shape of the pH curves in tris buffer found here and, in confirmation

of the work presented here, the pH optimum is in the vicinity of 8. Using the exact technique and methods of Sherry and Troll, the optimum was again found to be at pH 8.

Both trypsin and plasmin, which operate on the same substrates as thrombin ((6) and Table I) and hydrolyze TAME particularly well, show the same pH optimum and broad curve shape (6) as was found here for thrombin. Troll, Sherry, and Wachman (12) had reported that the pH optimum of plasmin acting on TAME was (as in their report of thrombin acting on TAME) at pH 9 in tris buffer. On the other hand, Ronwin reported the pH optimum of plasmin (6) when acting on TAME in tris, veronal, and borate buffers to be (as that reported here for thrombin) at pH 8. It is suggested that an explanation analogous to the above for the variation in optima is likely.

The broad pH optimum around pH 8 in veronal determined the choice of this buffer for all succeeding experiments.

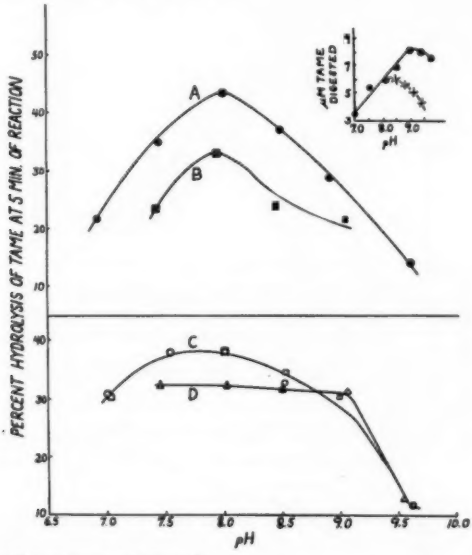

FIG. 1. The pH optimum of thrombin.

Standard conditions (see Materials and Methods section) except as indicated. All curves adjusted to an initial enzyme concentration of 3.80 Th units/ml. (the Th unit is defined in the text). A—0.015 *M* tris. B—0.25 *M* tris; S_0: 0.04 *M* TAME. C—0.015 *M* Veronal. D—0.015 *M* borate.

Curve in upper right hand corner reproduced from Sherry and Troll (11). ×-×-×-line added by author.

Definition of the Th Unit for Thrombin Activity

It is thought desirable to define a new unit for thrombin activity and to designate this as the Th unit. Sherry and Troll (11) have defined a unit of enzyme activity as follows: "that amount of thrombin which will release one micromole of acid from TAME in ten minutes *under the conditions of the test.*" However, the "conditions of the test" were not clearly defined.

These authors introduced variations in the physical conditions of their experiments when variation of these parameters was not the object of the experiment; thus one is left uncertain whether the conditions of the test require 0.01 *M*, 0.03 *M*, or 0.04 *M* TAME, 35° or 37°, 0.3 *M* or 0.25 *M* tris buffer.

Sherry and Troll base their unit on a linear hydrolysis of TAME with time (insert in Fig. 2). They specify that the micromoles of TAME digested in 10 minutes (their activity unit) should be determined by ascertaining the micromoles hydrolyzed in 30 minutes and dividing by 3 to obtain the 10 minute value. This practice may be satisfactory with a straight line function; however, the data of Fig. 2 demonstrate that the shape of the hydrolysis curve of TAME as a function of time is dependent upon enzyme concentration (and probably substrate concentration, too) and that the relationship is not necessarily linear over the entire course of the reaction.

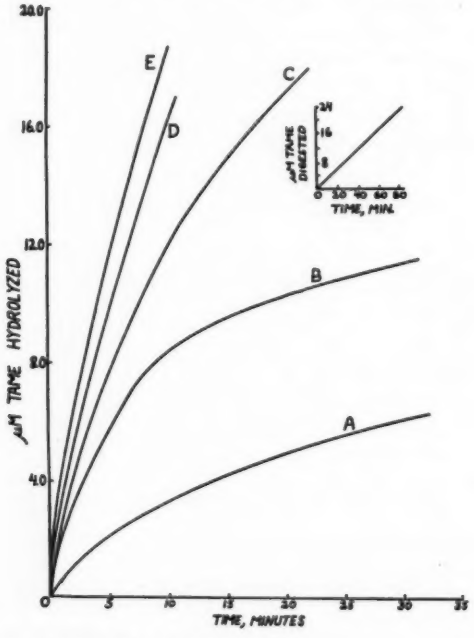

FIG. 2. Hydrolysis of TAME as a function of time.
Standard conditions (see Materials and Methods section).
Curve in upper right hand corner reproduced from Sherry and Troll (11). Initial enzyme concentrations as follows: A—1.27 Th units/ml. B—3.17 Th units/ml. C—3.80 Th units/ml. D—5.07 Th units/ml. E—6.33 Th units/ml.

The Th unit of thrombin activity is defined as follows: the unit of thrombin activity equals that potence of the enzyme which will release 1 micromole of acid from TAME per ml. of reaction mixture in 5 minutes using an initial

substrate concentration of 0.01 *M* TAME in 0.015 *M* veronal buffer at 38° C. and pH 8.0 ± 0.05, the reaction being conducted under nitrogen gas flush and followed by a continuous titration procedure.

Rate of Hydrolysis as a Function of Initial Enzyme Concentration

Fig. 3 presents the effect of initial enzyme concentration on the rate of TAME hydrolysis. The data demonstrate that over the sixfold range of enzyme concentration used, the rate of hydrolysis is proportional to this parameter.

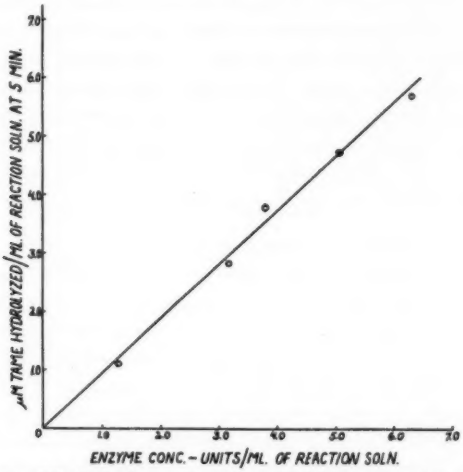

FIG. 3. The rate of hydrolysis as a function of initial enzyme concentration. Standard conditions (see Materials and Methods section).

Substrate Specificity of Thrombin and Comparisons with Related Enzymes

The action of thrombin on various arginine methyl ester derivatives is given in Table I. Comparisons of quantitative significance can only be made within a column; comparisons across columns have relative meaning only.

TABLE I

SPECIFICITY OF THROMBIN AND RATIO COMPARISONS WITH RELATED ENZYMES

Substrate	Thrombin		Plasmin,† ratio TAME/other cpd.	Trypsin,† ratio TAME/other cpd.
	% Hydrolysis at 5 min. of reaction*	Ratio TAME/other cpd.		
TAME‡	33.3			
AMED	0.0§			
BAME	11.6	2.9	6.7	11.1
3,5-DNBAME	4.3	7.8	4.4	12.4
MNBAME	3.3	10.0	8.1	20.0

*Standard conditions (see Materials and Methods section); rates corrected to an equal enzyme activity of 3.33 Th units/ml. of reaction solution.

†See reference (6).

‡For abbreviations see Materials and Methods section.

§No neutralization of added base observed after 75 minutes.

AMED is not acted upon by thrombin under the conditions employed here, though Sherry and Troll (11) report its hydrolysis at higher pH using roughly 45 times more thrombin. Plasmin had earlier been found to lack action on this substrate and, at pH 8, it proved to be an extremely poor substrate toward a high concentration of trypsin (6). While TAME is the best substrate of the group for all three enzymes, it is obvious that N-acylated arginine esters fit a general substrate specificity requirement. Though AMED is not in this category, it is proposed that both it and the acylated arginine esters permit of the same binding of the α-amino nitrogen atom through its two "extra" electrons to the same or similar positive entity on all three enzymes in addition to binding at the other two points which all the compounds in Table I have in common (see section on ES complex).

The ability of all three enzymes to attack the same substrate types suggests identical or near-identical active centers. Such variations as exist in the relative activity ratios are probably due to minor differences in the structural geometry and group identity of the active center or its environs.

Stability Studies on Thrombin: Storage at 0–4° C.

Fig. 4 presents a study of the stability of thrombin preparations when stored at 0–4° C. The medium for each preparation is given in the legend and note should be taken that the data have been corrected to equal aliquot activities drawn from thrombin solutions of equal volume. The single curve appears to represent well the enzyme activity trend for all three preparations. There is a gradual rise in enzyme potency for about 25 days after which a leveling off to a more constant value takes place. The rise probably reflects the conversion of prothrombin to thrombin. In general this initial rise can yield values of enzyme potency which reach as much as 100% above the original values of the preparation. From day to day the

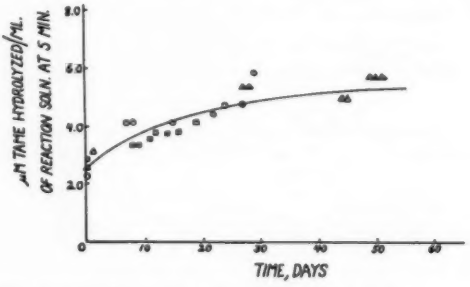

FIG. 4. Stability of thrombin preparations stored at 0–4° C.
Tests of activity performed under standard conditions and procedures (see Materials and Methods section). As presented all values are corrected to enzyme potencies for the condition of equal aliquots of original solutions of equal size.
○ One vial dissolved in 1 ml. of isotonic saline.
□ One vial dissolved in 1 ml. of 1 : 1 glycerol : H_2O.
△ One vial dissolved in 2 ml. of isotonic saline.
All vials contained Parke, Davis & Co., Lot no. 029342-B material and were labelled as having 1000 NIH units. From private communication, it was learned that each vial of this lot assayed at approximately 1300 NIH units and 7.52 mg. N per vial.

variation in enzyme activity is very slight. Nevertheless, quantitative studies required a daily check of activity in order to permit comparisons for the work of that day.

Stability Studies on Thrombin: Incubation at pH 8 and 38° C.

Fig. 5 presents another series of results obtained in stability studies with thrombin. A slow but definite decrease in the potency of thrombin takes place when it is incubated under the experimental conditions. For 10 to 15 minutes after the thrombin has been put into these media it can be used without much loss in activity. This closely approximates the behavior found for plasmin (6) under the same conditions and contrasts markedly with the rapid deterioration of trypsin under these same conditions (6). In 0.01 *M* citrate the enzyme inactivation is slowed; on the other hand, Ca^{++} accelerates thrombin decomposition. Addition of both cancels the effect of either, since the resulting enzyme activity degradation curve is well described by that obtained when neither of the salts is present. Ca^{++}-citrate complexing neutralizes the two ions.

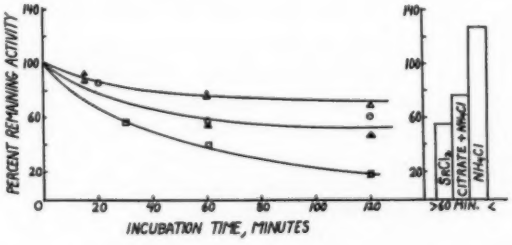

FIG. 5. Stability of thrombin incubated at pH 8 and 38°: influence of various ions on the incubated enzyme.

Experimental procedure

Twice the quantity of enzyme required for the test of its activity was incubated at pH 8 and 38° in 2 ml. of 0.015 *M* veronal buffer for intervals as indicated on the graph. At the end of the incubation period 1 ml. of the incubation solution was removed and pipetted into 1 ml. of substrate solution at such concentrations and conditions that standard conditions (see Materials and Methods section) were realized. Activities at 5 minutes of reaction were used for the comparisons made. It should be noted that the zero point is not a true zero, since it takes about 1 minute to inject the enzyme solution into the incubation medium using a micropipette, followed by thorough shaking and then by the withdrawal of a 1-ml. aliquot which is added to the milliliter of substrate solution intended for the test. The true zero, then, is actually a minute incubation period in most cases.

For curve section

○ No salts present in incubation medium. Enzyme concentration: 7.92 Th units/ml.
□ 0.01 *M* $CaCl_2$ in incubation medium. Enzyme concentration: 7.92 Th units/ml.
△ 0.01 *M* Na_3 citrate in incubation medium. Enzyme concentration: 8.32 Th units/ml.
▲ 0.01 *M* Na_3 citrate plus 0.01 *M* $CaCl_2$ in incubation medium. Enzyme concentration: 8.32 Th units/ml.

For column section

0.01 *M* NH_4Cl in incubation medium. Enzyme concentration: 8.64 Th units/ml.
0.01 *M* NH_4Cl plus 0.01 *M* Na_3 citrate in incubation medium. Enzyme concentration: 10.15 Th units/ml.
0.01 *M* $SrCl_2$ in incubation medium. Enzyme concentration: 8.64 Th units/ml.
The stated enzyme concentration refers to the concentration of the enzyme in the incubation mixture prior to the determination of enzyme activity remaining.

The results with Sr^{++} and NH_4^+ are shown at the right in Fig. 5. As with Ca^{++}, the activity drop occurs rapidly in the presence of Sr^{++}. With NH_4^+ a 26% increase in activity of the enzyme occurred after 60 minutes of incubation. Apparently, ammonium chloride hastens the conversion of some substance to thrombin. This finding differs from that of Seegars (10) to the effect that ammonium chloride has no activating influence. Perhaps as the result of an ammonium–citrate complex, incubation in the presence of both reduces the activating influence of ammonium chloride. These data assume interesting significance when it is noted (Table II) that NH_4^+, Ca^{++}, and Sr^{++} all activate the hydrolysis of TAME by thrombin. Thus, the NH_4^+ ion possesses at least two activating influences on the system of proteins involved in the blood clotting process, one mutual with Ca^{++} and Sr^{++} and one distinct from these alkaline earth cations.

Ware and Seegars (13) demonstrated a time lag of several hours between the disappearance of prothrombin and the corresponding increase in thrombin during prothrombin activation studies. This suggests that prothrombin may first be converted to an intermediate which might aptly be called *thrombinogen*. While citrate is known to activate the conversion of prothrombin to thrombin (10), it is likely that the decrease in thrombin inactivation in its presence (Fig. 5) is due to the functioning of the ion as a thrombin inhibitor (see Table IV) thus preventing thrombin's autohydrolysis. If citrate does activate some precursor to thrombin, this precursor may not be present in the thrombin preparation used or the concentration of citrate may have been too low (10). Further, it is unlikely that NH_4^+ and citrate, having, as they do, opposite charges, would activate the same precursors. Thus, a tentative reaction scheme is suggested:

$$X \xrightarrow[\substack{\text{enzyme (?)}\\ \text{citrate (?)}}]{(1)} \text{Prothrombin} \xrightarrow[\substack{\text{enzyme (?)}\\ NH_4^+ (?)}]{(2)} \text{Thrombinogen} \xrightarrow[\substack{\text{enzyme (?)}\\ NH_4^+ (?)}]{(3)} \text{Thrombin} \xrightarrow[\substack{\text{thrombin}\\ NH_4^+;\ Ca^{++};\ Sr^{++}}]{(4)} \text{Degraded Products}$$

Step 1 may represent more than one reaction. Whether NH_4^+ acts definitely at step 2 or step 3, or whether thrombinogen actually occurs, cannot be decided from the presently available data.

It should be noted that the actual activation caused by NH_4^+ is considerably more than 26% at 60 minutes of incubation. The NH_4^+ ion accelerates thrombin action somewhat less than Ca^{++} (Table II); consequently, it should cause the decomposition of thrombin (or reflect a loss in enzyme activity) at a level at least equal to that observed for incubations when no salt is present (40% loss in enzyme activity at 60 minutes, Fig. 5), and probably closer to that found when Ca^{++} is present (60% thrombin loss in 60 minutes, Fig. 5). Assuming a mid-point, the original thrombin should have lost 50% of its activity in the NH_4^+ incubation mixture at 60 minutes. Thus a finding of 126% activity compared to the "corrected" level indicates 76% activation, presumably of some precursor to thrombin, in the presence of NH_4^+.

The Effect of Ionic Strength on Thrombin Activity

Fig. 6 presents the data. Under standard conditions an ionic strength of 0.025 results from the presence of the substrate and the buffer.

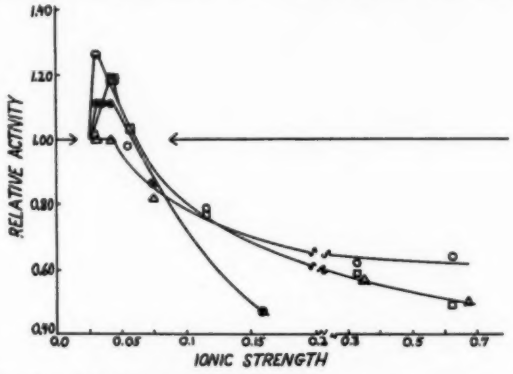

FIG. 6. The effect of ionic strength on thrombin.
Standard conditions (see Materials and Methods section), except for ionic strength, which was varied by introduction of appropriate salt concentrations to the ES reaction medium.
○ $SrCl_2$; enzyme concentration: 3.23 Th units/ml.
□ $CaCl_2$; enzyme concentration: 3.23 Th units/ml.
△ NaCl; enzyme concentration: 3.80 Th units/ml.
● NH_4Cl; enzyme concentration: 3.80 Th units/ml.
Activity taken as the percentage substrate hydrolyzed at 5 minutes of reaction.

Ca^{++}, Sr^{++}, and NH_4^+ all activate the thrombin hydrolysis of TAME but the activation is sensitive to ionic strength. In fact, an inhibition appears in all three cases at an ionic strength of 0.065 and increases with greater ionic strength. This inhibition might actually reflect an acceleration of thrombin autohydrolysis, the resulting enzyme loss registering as "inhibition." On the other hand, Na^+ has no activating influence, as was similarly found for most of the other ions listed in Tables II and IV, and begins to show significant inhibition (10% enzyme activity decrease) at ionic strengths greater than 0.05. Thrombin is significantly inhibited by all the salts tested at the ionic strength of physiological isotonic saline.

From Fig. 6 and Tables II and IV it can be concluded that inhibition arises in some cases from a specific action due to the nature of the ion (examples: Ni^{++}, Cu^{++}, Zn^{++}, and Cd^{++}; the ionic strengths in these experiments differed little from the base point) and in other cases (Mg^{++}, Li^{++}, $MoO_4^{=}$, and those given in Fig. 6) from the more general effect of ionic strength. This latter effect becomes significant between 0.035 and 0.055.

Action of Cations on Thrombin: Comparison with Related Enzymes

Table II contains a list of cations which were tested at two different concentrations for their effects on thrombin. The last three columns, in which salt concentrations and other physical conditions were the same, permit a qualitative but not quantitative comparison. Those cations which inhibit strongly

do so to all three enzymes, though the relative degrees of their effects differ with each enzyme. Cu^{++} is an extremely potent inhibitor of thrombin and the other enzymes. Ni^{++}, Zn^{++}, and Cd^{++} are again notable in their inhibition. At the lower concentration, with the exception of Be^{++}, the alkali and alkaline earth cations are either activating or without significant influence. With increased concentration, several of them become inhibitory, notably Mg^{++}. In both series, the first member of the group is the strongest inhibitor. Though Co^{++} and Fe^{+++} are members of the same chemical family as Ni^{++}, they are without influence at these concentrations in contrast to the potent inhibition by Ni^{++}.

TABLE II

ACTION OF CATIONS ON THROMBIN: COMPARISON WITH RELATED ENZYMES

	Thrombin						Plasmin,* relative activity	Trypsin,* relative activity
Salt	Conc. (*M*)	Ionic strength	Relative activity†	Conc.‡ (*M*)	Ionic strength	Relative activity†		
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
None		0.025§	1.00		0.025§	1.00	1.00	1.00
$BeCl_2$	0.0066	0.045	0.70	0.01	0.055	0.63		
$MgCl_2$	0.0066	0.045	1.00	0.01	0.055	0.78	1.09	
$CaCl_2$	0.0066	0.045	1.25	0.01	0.055	1.00	1.23	1.19
$SrCl_2$	0.0066	0.045	1.18	0.01	0.055	0.90		
$BaCl_2$	0.0066	0.045	1.00	0.01	0.055	0.95	1.06	
NH_4Cl	0.0066	0.032	1.11	0.01	0.035	1.11		
CH_3NH_3Cl	0.0066	0.032	1.00	0.01	0.035	0.90		
$(CH_3)_4NCl$	0.0066	0.032	1.00					
$(NH_4)_2SO_4$	0.0066‖	0.045	1.12	0.01¶	0.055	0.82		
$CuCl_2$	0.00066	0.027	0.04	0.001	0.028	0.00	0.31	0.03
LiCl	0.0066	0.032	1.00	0.01	0.035	0.80		
NaCl	0.0066	0.032	1.00	0.01	0.035	0.90		
KCl	0.0066	0.032	1.00	0.01	0.035	0.95	0.96	
RbCl	0.0066	0.032	1.00	0.01	0.035	0.93		
CsCl	0.0066	0.032	0.94	0.01	0.035	0.95		
$CoCl_2$	0.00033	0.026	1.00	0.0005	0.027	1.02	0.85	0.64
$FeCl_2$	0.00066	0.029	1.00	0.001	0.031	0.97	0.94	
$NiCl_2$	0.00066	0.027	0.63	0.001	0.028	0.59	0.42	0.58
$CrCl_2$	0.00066	0.029	1.00					
$ZnCl_2$	0.00066	0.027	0.88	0.001	0.028	0.71	0.41	0.12
$MnCl_2$	0.00066	0.027	1.00	0.001	0.028	1.00		
$CdCl_2$	0.00066	0.027	0.77	0.001	0.028	0.74	0.64	0.53
$HgCl_2$	0.000066	0.025	0.93	0.0001	0.025	1.00	0.97	0.88
$AgNO_3$	0.00033	0.025	0.90	0.0005	0.026	0.95	1.00	0.64

*See reference (6). The salt concentrations present in the plasmin and trypsin experiments were those given in column (5).

†Standard conditions (see Materials and Methods section); enzyme concentration: 2.85 to 4.13 Th units/ml. Rates compared at 5 minutes of reaction.

‡These concentrations were compatible with clear solutions at the start and end of the reaction, except in the case of $CrCl_2$ where some colloidal character accompanied the solution. In many cases, particularly with the heavy metal ions, these concentrations represent the maximum that will yield clear solutions throughout the reactions.

§Due to substrate and buffer.

‖Actually 0.0132 *M* with respect to NH_4^+.

¶Actually 0.02 *M* with respect to NH_4^+.

NH_4^+, Ca^{++}, and Sr^{++} show activation toward thrombin dependent on their concentration. The fact that Ca^{++} is an activator finds parallel in its similar behavior toward plasmin and trypsin. Methylammonium ion had no effect at the lower concentration and was somewhat inhibitory at the higher concentration. Significantly, tetramethylammonium ion at 0.0066 *M* was similarly without effect.

As a tentative explanation for the activation by Ca^{++} and Sr^{++}, it is suggested that the Ca^{++} ion may possess a proclivity to fill the *3d* level thereby forming activated co-ordinate complex type unions with the enzyme active center group (*s*). (Sc which follows Ca in atomic number fills the *3d* level in preference to the *4p* level). With Sr^{++} the analogous shell is *4d*. For Be and Mg no analogous subshell exists comparable to that in Ca^{++} and Sr^{++}. While Ba^{++} has a potential *5d* level, the situation is not analogous to Ca^{++} and Sr^{++} for, whereas the elements following Ca and Sr fill the respective *d* levels before any outer subshell, only La puts an electron in the *5d* level but is followed by Ce and the rare earth series which fill the *4f* level while carrying only *one* electron in the *5d* level. Thus any co-ordinate compound formed by Ca^{++} and Sr^{++} involving *two* or more electrons and utilizing their outer *d* level orbital possibilities would not be expected to form with Ba^{++}. The Ra situation is analogous to that of Ba.

As neither amount of charge nor ion size (4) relate Ca^{++} and Sr^{++} with NH_4^+, no proposal for a common mode of activation suggests itself. Further consideration of the action of NH_4^+ must be tempered by evidence presented in the next section which points to the ammonia molecule rather than the NH_4^+ ion as the activating agent.

Effect of pH on the Activation of Thrombin

Table III presents the data from this study. Ammonium chloride is pH dependent in its activation of thrombin; methylammonium chloride gives signs of behaving likewise and tetramethylammonium chloride lacks significant activating effect (less than 10%). While the activation by Ca^{++} and Sr^{++} is also pH dependent, that by ammonium chloride does not occur at pH 7.4 as is the case for the former ions. When the concentration of ammonia molecule begins to become significant, as at pH 8, ammonium chloride shows a degree of activation similar to that of the alkaline earths. While not excluding other possible explanations, the data do support the notion that the activating agent is the NH_3 molecule.

TABLE III

EFFECT OF pH ON THE ACTIVATION OF THROMBIN

Salt (0.0066 *M*)	Ionic strength	Relative activity at 5 min. of reaction* at pH: 7.0	7.4	8.0	8.5	9.0
None	0.025†	1.00	1.00	1.00	1.00	1.00
NH_4Cl	0.032	1.04	1.03	1.16	1.17	
CH_3NH_3Cl	0.032	1.00	1.06	1.04	1.04	1.09
$(CH_3)_4NCl$	0.032	1.03	1.02	1.00	1.04	1.06
$CaCl_2$	0.045	0.85	1.18	1.25	1.19	0.97
$CaCl_2$	0.035	0.95‡				
$SrCl_2$	0.045	0.89	1.15	1.18	0.83	0.87

*Standard conditions except pH as given (see Materials and Methods section); enzyme concentration: 3.33 to 3.80 Th units/ml.

†Due to substrate and buffer.

‡Salt concentration: 0.0033 *M*.

Since Ca^{++} and Sr^{++} ions are inhibitory at pH 7, they presumably are able to combine with a group at the active center at this pH. As the pH is raised to 7.4, significant activation is observed, which suggests that the already bound Ca^{++} and Sr^{++} ions enter into an additional complex with a second group that becomes available between pH 7.0 and 7.4. The active complex is apparently stable between pH 7.4 and 8.5. Above pH 8.5 at least one of the groups must be so affected as to hinder it from forming fruitful complexes with either ion, while complexing with the other group is still possible; witness the inhibition by Sr^{++} ions at pH 8.5 and by both Ca^{++} and Sr^{++} at pH 9.

Action of Anions on Thrombin

Table IV shows that most of the anions, regardless of charge or specific chemical nature, are inhibitory. Nitrate ion is fairly potent in its inhibition but nitrite ion lacks any effect. Similarly, dichromate inhibits significantly

TABLE IV

ACTION OF ANIONS ON THROMBIN

Salt (0.01 *M*)	Relative activity at 5 min. of reaction*	Initial ionic strength	Salt (0.01 *M*)	Relative activity at 5 min. of reaction*	Initial ionic strength
None	1.00	0.025†	$NaNO_2$	1.00	0.035
Na_2 succinate	0.88‡	0.055	$Na_2C_2O_4$ (oxalate)	0.75	0.055
Na_3 citrate	0.67	0.085	$NaKC_4H_4O_6$ (tartrate)	0.80	0.055
	0.80§, ‖	0.065	$Na_2S_2O_6$	0.86	0.055
Na_2SO_4	0.76	0.055	$Na_2S_2O_3$	0.72	0.055
	1.00‡, ‖	0.045	Na_2CrO_4	1.07	0.055
Na acetate	0.86	0.035	$Na_2Cr_2O_7$	0.78	0.055
NaF	0.74	0.035	Na_2HAsO_4	0.86	0.055
NaCl	0.90	0.035	Na_2WO_4	0.76	0.055
NaI	0.86	0.035	Na_2MoO_4	0.86	0.055
NaCN	0.76	0.035	$Na_2B_4O_7$	0.86	0.023¶
NaN_3	0.86	0.035	Na_2HPO_4	0.95	0.055¶
$NaNO_3$	0.76‡	0.035			

*Standard conditions (see Materials and Methods section); enzyme concentration, except as noted: 3.33 Th units/ml.
†Due to substrate and buffer.
‡Enzyme concentration: 3.80 Th units/ml.
§Enzyme concentration: 3.57 Th units/ml.
‖Salt concentration: 0.0066 *M*.
¶Salt doubled as buffer.

while chromate ion is devoid of such influence. Fluoride is more potent than its larger relatives. Of import are the inhibitory effects of citrate and oxalate, which are used as anticoagulants in blood sampling procedures. At higher ionic strengths both sulphate and thiosulphate show inhibition of very similar magnitude. That succinate at the same concentration as citrate is less inhibitory may have been expected in view of the previous discussion on the data of Fig. 6 concerning the effect of ionic strength.

Effect of Organic Reagents on Thrombin Activity

Table V is a compendium of the observations made on the inhibitory qualities or lack of these qualities by various organic agents which possess structural similarities to TAME or the other substrates listed in Table I.

Both L-arginine and L-arginine methyl ester proved inhibitory to the same degree. While the enzyme may be affected by their arginyl side chain, several of the other compounds possessing the arginyl side chain are not inhibitory, nor is the free guanidino group (as guanidine). L-Aspartic acid and its chloroacetyl derivative inhibit equally well. (Sherry and Troll (11) had found aspartic acid to be an inhibitor of the coagulating property.) These two compounds have in common, at the pH of the reaction, ionized α-carboxyl and β-carboxyl groups. From the lack of any effect by L-asparagine, which possesses the ionized α-carboxyl group but has an amide structure instead of the β-carboxyl group, it is reasonable to conclude that the ionized β-carboxyl group contributes to their inhibitory action. Further, as L-glutamic acid is without influence, it may be that both ionized carboxyl groups are necessary for inhibition but that they must be separated by just the distance that they are in the aspartyl compounds.

TABLE V

EFFECT OF ORGANIC REAGENTS ON THROMBIN ACTIVITY

Organic agent (0.01 *M*)	Relative activity at five minutes of reaction*
TAME substrate	
None	1.00
Taurine	1.00
L-Arginine hydrochloride	0.88
L-Arginine methyl ester $(HCl)_2$	0.90
Guanidine.HCl	1.00
L-Glutamic acid	1.00
L-Aspartic acid	0.88
N-Chloroacetyl-L-aspartic acid	0.88
L-Asparagine	1.00
N^{α}-Benzoyl-L-arginine	0.75
N^{α}-*m*-Nitrobenzoyl-L-arginine	0.85
N^{α}-*p*-Toluenesulphonyl-L-arginine	1.00
N-*p*-Toluenesulphonylglycine	0.94
Benzoic acid	1.00
p-Hydroxybenzoic acid†	0.94
p-Aminobenzoic acid	1.00
p-Nitrobenzoic acid	0.88
3,5-Dinitrobenzoic acid	0.88
BAME substrate	
None‡	1.00
N^{α}-Benzoyl-L-arginine‡	1.00
N^{α}-*p*-Toluenesulphonyl-L-arginine‡	1.04

*Standard conditions (see Materials and Methods section); unless otherwise noted, enzyme concentration: 3.80 Th units/ml.
†Enzyme concentration: 3.56 Th units/ml.
‡Enzyme concentration: 10.0 Th units/ml.

Consideration of the inhibition levels of benzoylated and *m*-nitrobenzoylated arginine coupled with those of the benzoic acid group indicates that inhibition in these cases is a function of the entire molecule.

Attention is drawn to the lack of inhibition by toluenesulphonyl-L-arginine or by toluenesulphonylglycine when TAME is the substrate. But, as mentioned above, benzoyl-L-arginine is a potent inhibitor when TAME is the substrate. On the other hand, both toluenesulphonyl-L-arginine and benzoyl-L-arginine are not inhibitory when BAME is the substrate. These results with benzoylarginine were previously observed with trypsin (6). Also, the inhibition by benzoylarginine along with lack of this effect by toluenesulphonylarginine when TAME was the substrate has been earlier demonstrated for plasmin (6).

It is permissible to become more general at this point than was possible in the previous report on plasmin and trypsin (6) by stating that, with enzymes possessing typical tryptic action, the amide grouping at the secondary peptide position of a trypsin (or thrombin or plasmin) substrate tends to increase the binding capacity of the compound toward the enzyme as compared to a sulphonylamide grouping at this position. Standing in sharp contrast is the observation that TAME, which has the sulphonylamide bond at the secondary peptide position, is hydrolyzed more rapidly than BAME, which has an amide at this same position. The significance of this result is discussed below.

The Thrombin ES Complex and a Consideration of Tryptic Enzyme Differences

The data accumulated thus far dictate a slight change in the previously proposed ES complex for trypsin and plasmin (6, 7, 8) and this changed complex is now assigned to all three enzymes (Fig. 7). The crux of the previously proposed theory (7, 8) that the weakening or rupture of the susceptible link is brought about by virtue of the dipositive-bond situation which arises at the bond as a result, in turn, of the manner of binding between the substrate and the enzyme remains unchanged.

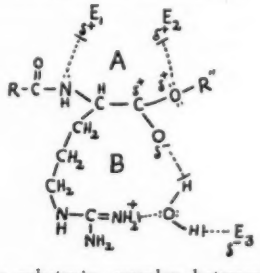

FIG. 7. Proposed enzyme–substrate complex between tryptic enzymes and their substrates.

In the proposed ES complex the α-nitrogen atom is pictured as complexing with the enzyme by virtue of its two "extra" electrons. The greater the acid strength of the acyl group attached to this α-nitrogen atom, the greater should be the pull on the "extra" electrons and, consequently, the less should be the tendency for the substrate to complex. However, once the ES complex does form, the greater the acid strength of that group, the greater will be the tendency for the susceptible link to cleave, since the acyl group will act to

make the carbon atom of the susceptible link more positive during the proposed dipositive-bond situation and therefore more susceptible to attack by nucleophilic agents such as hydroxyl ions.

Thus, the binding of the substrate and its subsequent hydrolysis are dependent in an opposite manner on the same force, namely, the acid strength of the substituent on the α-nitrogen atom (or equivalent group or atom) of arginyl (and probably, too, lysyl) esters. Ronwin has previously reported analogous data on carboxypeptidase and its acylated amino acid substrates which support this thesis (7, 9). The fact that benzoylarginine inhibits TAME hydrolysis by tryptic enzymes while toluenesulphonylarginine lacks such action is also consistent with the thesis, as is the greater susceptibility of TAME to these enzymes when compared to BAME. Further, the slow but definite hydrolysis of AMED by thrombin (11) and by trypsin (6) finds explanation in the thesis and proposed ES complex. At pH 8, and much more so at pH 9, some of the α-amino group of AMED would exist in the dissociated form, which would permit binding at this group to the enzyme in the same way as proposed for the acylated arginyl esters (Fig. 7). However, as an α-amino group bound as proposed would be nowhere as electron-pulling as a similarly bound toluene-sulphonamido or benzamido group, its influence in sustaining the positive nature of the susceptible link would be negligible and the tryptic hydrolysis of this compound, as would be expected, is very poor. Also, by virtue of its ability to bind to the enzyme, AMED, as arginine, should be, and has been shown here to be, an inhibitor.

Previous observations (6) combined with those reported here emphasize the extraordinary similarity between thrombin, plasmin, and trypsin; witness their action on the same substrates,[2] identical pH optimum curves, close linearity between enzyme concentration and reaction rates over wide concentration ranges, inhibition and activation by the same ions, and similar response toward organic agents, particularly benzoylarginine.

However, differences do occur. Thus plasmin and trypsin showed marked resistance to combined acid and heat treatment while thrombin rapidly deteriorated under the same conditions (6). At pH 8 and 38°, thrombin and plasmin slowly decompose while trypsin is quickly inactivated. Of course, the obvious clotting action of thrombin and the lack of this action by the other two enzymes is a most important difference. In general, such differences as do exist seem most marked in relation to changes in physical milieu.

Therefore, it becomes most tempting to propose that the active centers of all three enzymes consist of two positive charges and one negative charge (Fig. 7) and have the very same groups responsible for these charges, but

[2]The report by Sherry and Troll (11) that thrombin is different from trypsin and plasmin because thrombin does not act on lysine esters, while trypsin and plasmin do, must be considered with caution since as explained above for AMED, lysine ethyl ester (the substrate used by Sherry and Troll) would be expected to exhibit very little or no substrate susceptibility. Had these authors used an acylated lysine ester they would probably have found considerable thrombin action as has already been demonstrated with trypsin (1, 2) for acylated lysine amides (which, if anything, would be hydrolyzed by tryptic enzymes much more slowly than the corresponding esters).

that surrounding groups and physical geometry are somewhat different and cause the observed differences.

This April, evidence was obtained for the existence of *thrombinogen* as proposed herein. The classic equation for the production of thrombin now appears more likely to proceed as follows (no Ca^{++} ion necessary):

$$\text{Prothromin} \xrightarrow{\text{Thromboplastin}} \text{Thrombinogen} \xrightarrow{\text{Thrombin}} \text{Thrombin}$$

Acknowledgment

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THE UTILIZATION OF ACETATE BY LIVER AND ADIPOSE TISSUE OF GROWTH HORMONE TREATED RATS OF DIFFERENT AGES[1]

W. F. Perry and Helen F. Bowen

Abstract

The effect of growth hormone on the *in vitro* incorporation of C^{14} acetate into fatty acids, carbon dioxide, and cholesterol by liver and adipose tissue from young, adult, and old rats was studied.

In all three age groups of animals, growth hormone was found to depress the incorporation of acetate into fatty acids by liver slices but the CO_2 production was unaffected. In both young and old animals growth hormone did not significantly alter the incorporation of acetate into fatty acids and CO_2 by preparations of adipose tissue, but did result in a decline in the fat content of the adipose tissue. It was noted that the CO_2 production from acetate was much less with adipose tissue from old rats than with similar preparations from young rats.

Incorporation of acetate into cholesterol was unaffected by growth hormone in young and old animals but was significantly increased in liver slices from adult animals.

Introduction

The metabolism of lipids in intact animals or isolated tissues can be influenced by nutritional and hormonal factors. For example, in fasting animals hepatic synthesis of fatty acids, cholesterol, and phospholipids is depressed (6) and in the adrenalectomized animal fatty acid and cholesterol synthesis is lower than in normal animals (10), as, also, is phospholipid synthesis (7). In the diabetic animal, although fatty acid formation is depressed, cholesterol synthesis is either unchanged (13) or actually increased (5). Thus, changes in fatty acid formation need not necessarily be paralleled by like changes in the formation of cholesterol or phospholipids. Growth hormone has been shown to inhibit acetate incorporation into liver fatty acids (9) and to inhibit the incorporation of deuterium into both carcass and liver fats (14). However, it has been reported that growth hormone does not depress cholesterol synthesis by the liver and may even enhance the process (1).

Recently we demonstrated a difference between very young and very old rats as regards acetoacetic acid formation from octanoic acid by the isolated rat liver slices following injection of growth hormone (11). As the demonstration of an inhibitory action of growth hormone on hepatic lipogenesis had been performed on adult animals, it was decided, in view of the differences in ketogenesis in young and old animals treated with growth hormone, to investigate the action of growth hormone on hepatic lipogenesis in very young and in aged rats. At the same time it was decided also to investigate a possible action of growth hormone on hepatic cholesterogenesis. A comparison of lipogenesis by adipose tissue in normal and growth hormone treated animals

[1]Manuscript received May 1, 1957.
Contribution from the Department of Physiology and Medical Research, University of Manitoba, Winnipeg, Manitoba.

was also undertaken as it has been shown that under certain circumstances, such as adrenalectomy (12) or hypoglycemia (8), changes in hepatic lipogenesis are not paralleled by qualitatively similar changes in lipogenesis in the depot fats.

Methods

The experiments were done with Wistar strain albino rats fed ad libitum on Purina Fox Food. The animals were classified as to age as young (1–1½ months old) or old (2+ years old). In addition, some experiments were also done on a smaller group of adult (4–5 months old) animals. The growth hormone used was Connaught Laboratories preparation dissolved in alkaline solution, pH 8.0, immediately before injection. It was injected intraperitoneally in 5 mg. doses.

Four hours after injection the treated animals and control, uninjected animals were killed by decapitation. The liver and intestinal mesentery, the latter being used as a source of adipose tissue, were quickly removed and placed in ice-cold buffer. Liver slices, 1 to 1·5 g., were quickly prepared by use of a hand microtome and suspended in 10 ml. of buffer at pH 7.4. For studies on hepatic lipogenesis and $C^{14}O_2$ production, a bicarbonate buffered medium, pH 7.4, containing K^+, Na^+, Mg^{++}, and Ca^{++} in concentrations of 70, 76, 6, and 2 meq./l. respectively, was used. The medium was supplemented with 2-C^{14} sodium acetate, 4 microcuries/ml., the total concentration of acetate in the medium being 1.5 μM./ml. For studies on hepatic cholesterogenesis, 1–1.5 g. liver slices were suspended in a phosphate buffer, pH 7.4, containing K^+, Na^+, Mg^{++}, and Mn^{++} in concentrations of 70, 76, 6, and 1 meq./l. respectively. The medium was supplemented with 2-C^{14} acetate, 4 μc./ml. (total acetate concentration 1.5 μM./ml.).

The adipose tissue was placed in 10 ml. bicarbonate buffer supplemented with succinate and glucose, as described by Feller (2), and 1-C^{14} acetate, 4 μc./ml. (total acetate concentration 1.5 μM./ml.).

Flasks containing bicarbonate buffer were gassed with 95% O_2 + 5% CO_2; those containing phosphate buffer, with 100% O_2. All flasks were incubated with shaking for 3 hours at 37° C., provision being made to trap the metabolic $C^{14}O_2$ produced during the incubation. At the end of this time the reactions were stopped by the addition of 1 ml. of 5 *N* HCl to the media. The tissues were then hydrolyzed with alcoholic potassium hydroxide and the fatty acids and cholesterol isolated.

Fatty acids were counted as plates corrected to a weight of 15 mg., cholesterol as infinitely thin plates of cholesterol digitonide. The trapped $C^{14}O_2$ was precipitated with barium chloride and infinitely thick plates of the barium carbonate resulting were counted.

Aliquots of adipose tissue were taken for nitrogen analysis by the micro Kjeldahl method, and the radioactivity of fatty acids isolated from the adipose tissue and $C^{14}O_2$ produced were expressed as c.p.m. per mg. adipose tissue nitrogen following the suggestion of Hausberger (4).

TABLE I

INCORPORATION OF 2-C^{14} ACETATE INTO CHOLESTEROL BY LIVER SLICES OF GROWTH HORMONE TREATED RATS

Mean values ± S.E.

Differences between controls and growth hormone treatment treated as paired data

		Control			Growth hormone		
	No.	Cholesterol, mg./g. liver	c.p.m. g. liver	S.A. c.p.m./mg. cholesterol	Cholesterol mg./g. liver	c.p.m. g. liver	S.A. c.p.m /mg. cholesterol
Young	8	1.82	6320 ±1260	3550 ± 820	1.86	4890 ±1700	3020 ± 855
				$P = > .05$			
Adult	5	1.60	5640 ± 960	3520 ± 710	1.65	9120 ±1510	5530 ±1415
				$P = < .05$			
Old	9	1.62	2850 ± 470	1668 ± 370	1.76	3170 ± 610	1810 ± 430
				$P = > .05$			

TABLE II

INCORPORATION OF 2-C^{14} ACETATE INTO FATTY ACIDS AND CO_2 BY LIVER SLICES FROM GROWTH HORMONE TREATED RATS

Mean values ± S.E.

Differences between controls and growth hormone treatment treated as paired data

		Control				Growth hormone			
		Fatty acids			CO_2	Fatty acids			CO_2
	No.	% fat	c.p.m. g. liver	S.A. c.p.m./15 mg. fat	c.p.m. g. liver	% fat	c.p.m. g. liver	S.A. c.p.m./15 mg. fat	c.p.m. g. liver
Young	6	2.9	7880 ±1600	7530	12,300 ± 1770	3.0	3400 ± 890	3230	11,150 ± 620
			P = < .01						
					P = > .05				
Old	8	3.0	7000 ±1000	6030	10,370 ± 1250	3.2	3310 ±860	2510	9530 ±1510
			P = < .02						
					P = > .05				

TABLE III

INCORPORATION OF 1-C^{14} ACETATE INTO FATTY ACIDS AND CO_2 BY ADIPOSE TISSUE OF GROWTH HORMONE TREATED RATS

Mean values—c.p.m. per mg. adipose tissue N ± S.E.

Differences between controls and growth hormone treatment treated as paired data

		Control				Growth hormone			
		Fatty acids			CO_2	Fatty acids			CO_2
	No.	% fat	c.p.m.	S.A. c.p.m./15 mg. fat	c.p.m.	% fat	c.p.m.	S.A. c.p.m./15 mg. fat	c.p.m.
Young	9	42 ± 3.0	3020 ± 490	1470	2490 ± 600	31 ± 3.5	3350 ± 410	2670 ±1320	1860 ± 400
					$P = < .05$				
					$P = > .05$				
									$P = > .05$
Old	9	70 ± 1.6	2180 ± 710	450 ± 130	358	62 ± 2.2	1970 ± 620	400 ± 150	364 ± 160
					$P = < .01$				
					$P = > .05$				
									$P = > .05$

In analyzing the results statistically, tissues from control and growth hormone treated animals examined on the same occasions were treated as pairs and the differences between pairs calculated and their significance determined.

Results

In Table I are recorded data on the incorporation of 2-C^{14} acetate into liver cholesterol. Administration of growth hormone did not result in any appreciable differences in the synthesis of cholesterol by the liver slices of young or old rats. In adult rats the activity of the cholesterol from growth hormone treated animals was significantly higher than that of the controls, as was the specific activity of the cholesterol. There is thus some indication that in adult rats growth hormone may stimulate hepatic cholesterogenesis. It was also apparent that the ability of the liver slices from old rats to incorporate acetate into cholesterol was less than that of young or adult rats in both control and hormone treated animals. However, untreated young and adult animals did not differ significantly in this respect.

In Table II the incorporation of 2-C^{14} acetate into fatty acids and CO_2 by liver slices from young and old rats is summarized. It will be seen that under the influence of growth hormone there was a decrease in the amount of acetate carbon incorporated into liver fatty acids. These findings are similar to those which we previously reported for adult animals (9). There was no significant difference noted in the $C^{14}O_2$ of liver slices between growth hormone treated and control animals, whether young or old; also no differences were noted in $C^{14}O_2$ production or fatty acid incorporation between control animals of either age group.

In Table III the action of growth hormone on lipogenesis by adipose tissue in young and old rats is compared. In contrast to the findings with liver tissue, there was no significant difference noted between the treated and control animals as regards acetate incorporation into fatty acids by preparations of adipose tissue; nor was there any real difference in $C^{14}O_2$ production from acetate in the hormone treated animals of either age group. However, in both young and old animals hormone treatment led to a decrease in the fat content of the adipose tissue. It was also observed, when young and old animals were compared, that the mesentery of young rats contained a lesser amount of fat on a wet weight basis and that acetate incorporation into CO_2 was much greater in the young animals. Incorporation of acetate into fatty acids was also greater in the young animals but this increase did not prove to be statistically significant. The specific activity of the fatty acids did suggest increased acetate utilization by young adipose tissue.

Discussion

It would seem that some effects of growth hormone administration depend on the age of the animal. Hepatic cholesterogenesis is unaffected by growth hormone in young and old animals but is slightly stimulated in the adult animal. However, the process of hepatic fatty acid synthesis is inhibited in

all age groups. Fatty acid synthesis in the depot fats, however, appears to be unaffected by growth hormone in young and old animals. In the liver, where fatty acid synthesis was depressed by growth hormone in all age groups, there was also a tendency for a decreased incorporation of acetate into CO_2, which decrease, however, did not prove to be significant. Administration of growth hormone in both young and old animals led to a decrease in the fat content of mesenteric tissue. This is probably related to the reported fat mobilizing action of growth hormone (3).

The fat content of young adipose tissue was much less than that of old tissue. This, together with increased hepatic cholesterogenesis and CO_2 production in young compared with old animals and increased production of CO_2 by young adipose tissue, suggests that certain pathways of acetate metabolism are more active in young animals. Age differences in ketogenesis by liver slices between young and old animals have been previously noted, in that growth hormone is ketogenic in young but not in elderly rats (11).

Hepatic cholesterogenesis was slightly stimulated in adult rats. This finding is in agreement with that of Allen, Medes, and Weinhouse (1), who, using adult rats only, reported increased acetate incorporation into cholesterol by liver slices of growth hormone treated animals, but also noted that the effect was not consistent. No effect of growth hormone was noted in our experiments on cholesterogenesis in slices from young or from very old rats.

Failure to demonstrate an effect of growth hormone on adipose tissue lipogenesis is not in agreement with Welt and Wilhelmi's demonstration (14) of an inhibitory action of growth hormone on deuterium incorporation into carcass fats in the intact animals. However, it does not necessarily follow that factors affecting hepatic lipogenesis should similarly affect lipogenesis in other tissues. It has been shown, for example, that the depressed hepatic lipogenesis in the adrenalectomized animal is not accompanied by a similar depression in the lipogenesis in the depot fats but, on the contrary, the rate of lipogenesis may be increased (12).

Although no clear pattern is apparent in the above data, it would appear that in an assessment of the influence of growth hormone on fat metabolism, the age of the animals should be taken into consideration. The growth hormone effect that seems to be common to all age groups is an inhibition of the process of incorporation of acetate into liver fatty acids and lack of effect on adipose tissue.

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MARINE FISH MUSCLE NUCLEIC ACIDS[1]

H. M. BLUHM and H. L. A. TARR

Abstract

The muscles of six species of North Pacific ocean fish contained from 42 to 142 μg. per 100 mg. wet weight of ribonucleic acid (RNA) and from 0.2 to 2.5 μg. per 100 mg. of deoxyribonucleic acid (DNA). The molar ratio of purine and pyrimidine bases in lingcod and sockeye salmon muscle RNA was: adenine, 1.0; guanine, 2.0; cytosine, 1.0; and uracil, 1.25. Chemical analysis of isolated lingcod muscle RNA indicated that it was a comparatively pure product and was free, or practically free, from protein and amino acids.

Introduction

The occurrence of free ribose in fish muscles stored at 0° C. for several days *post mortem* was first demonstrated in this laboratory (10), and the role of riboside hydrolase enzymes in its formation was established later (11, 12). During these investigations it was observed that alcoholic extracts of *post rigor* marine fish muscles contained significant quantities of ribonucleosides which were not evident in muscles of freshly killed fish. The present paper records the quantitative distribution of RNA and DNA in the muscles of six species of North Pacific ocean fish, and describes the isolation and chemical characterization of RNA from sockeye salmon and from lingcod.

Materials and Methods

Whole small fish, or pieces of excised muscle from larger fish, were frozen in an insulated box containing crushed solid carbon dioxide as soon after capture as possible and normally while the fish were still alive. The samples were wrapped in polyethylene and stored at −25° C. until they could be used conveniently.

While still frozen, samples of the muscles were homogenized in cold 70% ethanol and fractionated by the method of Ogur and Rosen (6). Quantitative determinations of the ribose (5) and deoxyribose (8) content of the appropriate fractions were made, and the RNA and DNA content of the tissues were calculated from these results. After several attempts to extract RNA from the frozen muscle samples by available methods it was found that only that of Volkin and Carter (14) yielded desirable products. The actual yields of RNA by this technique were, as stated by the above authors, comparatively poor, amounting to only 20–25% of the calculated RNA content of the muscles. However, when freeze-dried from aqueous solution, the sodium RNA was obtained as a fluffy, white, completely water-soluble product of high purity (see below).

For determination of the purine and pyrimidine base content the RNA samples were hydrolyzed 1 hour at 100° in 70% perchloric acid (Wyatt (15)). The free bases were separated by quantitative paper chromatography using

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isopropanol:HCl:H_2O solvent (15) in 16-hour ascending development at room temperature. The compounds were located on the air-dried chromatograms with a "Mineralite" ultraviolet lamp, and their concentrations determined by elution with 0.1 *N* HCl for 18 hours at room temperature and application of the differential ultraviolet spectrophotometric method of Vischer and Chargaff (13) to the eluates. The nitrogen content of purified lingcod RNA was determined by the micro-Kjeldahl procedure and the phosphorus content by the method of Fiske and Subbarow (1). A quantitative biuret test (3) of the lingcod RNA and chromatography of its acid hydrolyzates (7) were employed in attempts to demonstrate the presence of protein and amino acids in the respective materials.

Results and Discussion

The RNA content of the fish muscles examined (Table I) was somewhat lower than that which has been reported for certain normal mammalian muscles (4), but the manner in which the samples were secured may have permitted some degradation. The DNA content was considerably below that which has been reported for mammalian skeletal muscles (4).

The molar ratio of purine and pyrimidine bases of the RNA prepared from lingcod and sockeye salmon muscles (Table II) was practically identical, being approximately as follows: adenine, 1.0; guanine, 2.0; cytosine, 1.0; and uracil, 1.25. The rather high guanine content is in keeping with results obtained with the RNA from carp muscle nucleotropomyosin (2). However, the cytosine content was considerably higher, and the uracil content somewhat lower, in the latter preparation, in which the molar ratio of the bases was as follows: adenine, 1.0; guanine, 2.1; cytosine, 1.85; and uracil, 1.1.

TABLE I

NUCLEIC ACID CONTENT OF FISH MUSCLES (μG./100 MG. WET WEIGHT)

	RNA	DNA
Sockeye salmon (*Oncorhynchus nerka*)	100	2.5
Spring salmon (*Oncorhynchus tschawytscha*)	142	1.4
Pink salmon (*Oncorhynchus gorbuscha*)	42	0.2
Coho salmon (*Oncorhynchus kisutch*)	123	1.5
Lingcod (*Ophiodon elongatus*)	47.3	1.0
Starry flounder (*Platichtys stellatus*)	58.0	2.0

TABLE II

MOLAR RATIO OF PURINE AND PYRIMIDINE BASES IN FISH MUSCLE RNA

	Sockeye salmon	Lingcod
Adenine	1.0	1.0
Guanine	1.95	2.3
Cytosine	1.08	0.97
Uracil	1.23	1.27

The results of detailed chemical analysis of lingcod RNA are recorded in Table III. The ratio of purines to pyrimidines is somewhat higher, and of nitrogen to phosphorus somewhat lower, than has been recorded for yeast RNA (9). Neither protein nor chloride could be detected in the preparation, and only minute traces of amino acids other than glycine, which is derived from acid hydrolyzed purines, were found in treated (7) preparations.

TABLE III

COMPOSITION OF LINGCOD SODIUM RNA

	μg./mg.	μM./mg.		μg./mg.	μM./mg.
Adenine	65.1	0.481	Chloride	Negative	
Guanine	168.6	1.115	Biuret	Negative	
Cytosine	52.3	0.471	Amino acids	Slight traces on hydrolysis	
Uracil	68.6	0.612	N/P ratio	1.68	
Purines	234.0	1.59	Purine/pentose	1.04	Theory 1.0
Purine bound ribose	228.0	1.52	Bases/phosphorus	0.94	" 1.0
Total bases	355.0	2.679	Base N/total N	1.01	" 1.0
Phosphorus	89.0	2.87	Purines/pyrimidines	1.46	
Nitrogen	150.0	10.7			
Nitrogen calculated from bases	149.0	10.6			

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